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Genetics and applications of nisin production in Lactococcus lactis subsp. lactis and conjugal exchange of this trait

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GENETICS AND APPLICATIONS OF NISIN PRODUCTION IN LACTOCOCCUS LACTIS SUBSP. LACTIS AND CONJUGAL EXCHANGE OF THIS TRAIT

by

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Jeffery R. Broadbent

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ABSTRACT

Genetics and applications of nisin production in *Lactococcus lactis* subsp. *lactis* and conjugal exchange of this trait.

by

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Chapter I reviews current literature on gene transfer systems in lactic acid bacteria, how genetically altered microorganisms for food are presently regulated, and how nisin is used as a food preservative.

Chapter II investigates previous reports which linked genes for nisin biosynthesis and sucrose utilization (Nip^+Suc^+) to plasmid DNA in two well characterized *L. lactis* subsp. *lactis* strains. Plasmid curing studies, conjugations, and DNA-DNA hybridizations indicated that these genes were encoded by chromosomal loci in all Nip^+Suc^+ strains examined. Similar results were noted in nisin-sucrose transconjugants of *L. lactis* subsp. *cremoris* and *S. salivarius* subsp. *thermophilus* in Chapters III and IV.

Chapter III describes the use of conjugation to construct nisin-producing Lactococcus lactis subsp. cremoris strains. The direct-plate conjugation method was developed to facilitate transfer of Nip^+Suc^+ to L. lactis subsp. cremoris recipients. DNA-DNA hybridizations to transconjugant DNAs with an oligonucleotide that detected the nisin structural gene, nisA, demonstrated that this gene was transferred during conjugation. Lactococcus lactis subsp. cremoris Nip^+Suc^+ transconjugants retained the recipient strain phenotype with respect to bacteriophage resistance and acid production in milk. These results indicated that it would be feasible to construct nisin-producing L. lactis subsp. cremoris strains for mixed and multiple starter systems.

Chapter IV investigates features of Nip^+Suc^+ transfer using a Lactococcus lactis subsp. lactis model system. Intergeneric transfer of nisin-sucrose genes was also achieved between lactococcal Nip^+Suc^+ donors and Streptococcus salivarius subsp. thermophilus recipients. Streptococcal transconjugants acquired Suc^+ and nisin immunity but did not produce nisin. DNA-DNA hybridizations, however, demonstrated that nisA was present in these transconjugants. To investigate whether nisA was involved in nisin immunity, this gene was cloned and electro-transformed into Lactococcus lactis subsp. lactis LM0230. Electro-transformants did not express nisin

immunity or any other trait linked to nisin production in lactococci.

Results presented in Chapter V indicate that nisin may have application for control or prevention of bovine mastitis. Gram-positive pathogens which cause bovine mastitis were examined for their susceptibility to nisin. Disc diffusion assays indicated that minimum inhibitory concentrations of nisin ranged from 10 to 250 ug per ml. In addition, 50 ug of nisin per ml in milk inhibited all gram-positive pathogens tested. (194 pages)

CHAPTER I

INTRODUCTION

Lactic Acid Bacteria. The lactic acid bacteria utilized around the globe to produce fermented dairy, meat and vegetable products include members from the genera Lactococcus, Lactobacillus, Leuconostoc, Pediococcus, and Streptococcus. These acid tolerant microorganisms produce copious amounts of lactic acid from glucose fermentation and significantly lower the pH of the fermented product. Because they lack respiratory capability, lactic acid bacteria synthesize lactate from glucose even in the presence of oxygen (121). Lactic fermentation of raw agricultural products yields foods with improved flavor, texture, nutritional value, and preservation qualities (40,72,121). Because of the important contributions these microorganisms provide to the human diet, considerable scientific interest has been focused upon the microbiology and genetics of lactic acid bacteria, especially those involved in milk fermentation.

It is likely that fermented dairy products have been part of the human diet since the time milk was first collected in containers and stored. Over the centuries these fermentations evolved into the unique cheeses, yogurts, and buttermilks with which we are familiar today. It was not until the 1900s, however, that commercial manufacturers of these products recognized that substantial

improvements in product consistency and quality were gained from the use of well characterized starter cultures (31). Since this development, the economic value of fermented dairy products has grown to represent approximately one fifth of the world total for all fermented foods including alcoholic beverages (112,126). Propagation of this important economic resource has relied upon modern microbiology and fermentation technology to consistently produce uniform, high quality products. Manufacturers have found that these properties are largely reliant upon the starter cultures utilized in the fermentation. As in the past, the key to continued viability of this valuable economic resource in the future will be starter cultures with known, predictable, and stable characteristics.

Biotechnology now offers investigators powerful tools to both firmly establish these qualities among cultures and to amend other traits of dairy microorganisms. Increased quality, decreased production and storage losses, and an expanded diversity of dairy products in the marketplace are examples of how biotechnology may contribute to a sound economic future for the dairy industry.

Gene Transfer Systems. Modern biotechnology is rooted within the ability to genetically manipulate living cells to heritably alter the physiological properties of the organism. This technology is possible because of the discovery of various mechanisms which permit the

introduction of exogenous DNA into the cell. Four gene transfer processes have been discovered and developed in lactic acid bacteria. These include transduction, conjugation, protoplast fusion, and transformation.

Transduction. Transduction is a bacteriophage mediated form of gene transfer which involves the inadvertent packaging of host DNA within a phage particle during viral replication followed by injection into, and expression of that DNA in a new host (121). The first report of this phenomenon appeared in 1952 when Zinder and Lederberg (145) noted that phage P22 transferred the ability to synthesize amino acids into auxotrophic Salmonella spp. Within lactic acid bacteria, transduction was discovered during the early 1960s (3,110), and became the first form of gene transfer available within these microorganisms. Although the first reports described transduction of chromosomal genes among lactococci (formerly classified as streptococci) by virulent bacteriophage, later investigations have usually employed temperate phage to transduce both chromosomal and plasmid DNA (26). Except for one report of plasmid DNA transduction by virulent bacteriophage among Streptococcus salivarius subsp. thermophilus (88), genetics studies of transduction within the lactic acid bacteria have focused upon lactococci where it has been used to realize significant advancements.

The first evidence to solidly link a metabolic capability in lactococci (lactose utilization and proteinase activity) to plasmid DNA was obtained by transduction (80,84). This was an important result because it revealed a biological basis for the instability of important fermentative traits which had been recognized among dairy starter cultures. Loss of plasmid DNA results in the concomitant and permanent loss of any traits which were encoded by that plasmid. Under most conditions, however, maintenance of plasmid DNA is not essential to cell survival. Thus, if an error occurs during cell division and a daughter cell does not acquire a particular plasmid, it may continue to grow and perhaps even predominate over the wild type.

Transduction also provided a strategy for construction of the first genetically improved lactococcal strain. McKay and Baldwin (81) isolated transductants in which the lactose and proteinase genes had integrated into the chromosome and demonstrated dramatically increased stability of these traits. Kempler et al. (56) subsequently utilized these transductants in cheesemaking trials and found that they produced Cheddar cheese with less bitterness defect than was obtained with the parental strain. Because transductants with chromosomally integrated genes exhibited about one-half the proteinase activity of the parental strain, these trials established

the first relationship between gene dosage and the organoleptic properties of a fermented food.

In conclusion, as the first form of gene transfer to be discovered among lactic acid bacteria, transduction proved very effective to establish important genetics principles among lactococci. It was employed to confirm the presence and importance of plasmid DNA within these bacteria and to demonstrate real avenues for the genetic improvement of dairy starter cultures. Further investigations of transduction within all lactic acid bacteria may provide additional information, such as phage mechanisms for chromosomal integration or new plasmid encoded functions, which could facilitate strain improvements.

Conjugation. Conjugation among bacteria is a natural form of gene transfer that requires physical contact between viable donor and recipient cells. A sequential model for the physical events involved in conjugal transfer emerged from studies focused principally upon transfer of the fertility (F) plasmid in Escherichia coli (13,53,141). In simplest terms the steps may be divided into 3 parts; stable cell-cell pair formation, DNA exchange, and resolution of the mating pair.

Formation of stable cell-cell contact between most gram-negative donor and recipient bacteria requires sex pili which are produced by the donor cell (53). Donor and

recipient aggregation between gram-positive cells must involve distinct mechanisms because these cells do not produce pili. Studies of Enterococcus faecalis have demonstrated that the exchange of conjugative plasmids which encode hemolysin is often mediated by specific sex pheromones produced by recipient cells. The pheromones are small, target-specific peptides which trigger synthesis of proteins, from conjugative plasmids in donor cells, required for conjugal exchange (14,24). Among the substances produced in response to the pheromone is an aggregation substance that facilitates stable cell-cell pair formation. Recent evidence has suggested that conjugal transfer of lactose utilization among Lactococcus lactis subsp. lactis may possess features similar to those of the Enterococcus faecalis system, such as the synthesis of aggregation substance (36,135,139). The production of pheromones, however, has not been reported and the events involved in stable pair formation remain poorly understood among lactic acid bacteria and other gram-positive organisms.

Even less understood among gram-positive bacteria are the molecular events which follow stable pair formation. Data obtained from studies of gram-negative bacteria indicate that DNA transfer occurs in single stranded form and is initiated at a specific locus designated the origin of transfer (oriT) (141). Transfer is followed by

complementary strand synthesis in the recipient cell and dissociation of the mating pair.

Conjugation among lactic acid bacteria was first discovered in lactococci and reported independently by Gasson and Davies (38) and Kempler and McKay (57), each of whom noted transfer of lactose fermenting ability. Although investigators have demonstrated conjugation of a few broad-host range plasmids and chromosomally encoded transposons within several species of lactic acid bacteria (26,39,49,85,94,100,128,138), most of the detailed information acquired to date has been derived from studies of lactococcal conjugation.

Among lactococci, conjugation has proven very useful for studies of plasmid biology and genetics (26,36,64). One important result of these studies has been the discovery that many industrially important traits of lactococcal starter cultures, such as the utilization of lactose and casein (38,39,57), bacteriophage resistance (12,59,82), and production of bacteriocins (35,93), are transmissible (for reviews see 26,36). This fortunate situation is of great practical significance to the biotechnological improvement of these organisms. Because conjugation occurs naturally between these food grade organisms, lactic acid bacteria which are genetically improved by this technique bypass many of the obstacles associated with the industrial application of strains which

contain recombinant DNA (26,109). As a consequence, conjugation offers the potential to genetically improve strains for industrial applications which merit almost immediate regulatory approval. Sanders et al. (109) utilized this strategy to effectively improve bacteriophage resistance among industrial strains of Lactococcus lactis.

Few conjugative traits have been identified among lactic acid bacteria other than lactococci, but within a few species of the lactobacilli used in dairy fermentations investigators have found conjugal transfer of lactose fermenting ability (11) and bacteriocin production (58). Of related significance have been reports of interspecific and intergeneric conjugal exchange among lactic acid bacteria (18,36,49,100,128,138). While these reports have principally involved transfer of broad host range self-transmissible plasmids which encode antibiotic resistance, rather than genes useful to industry, they have demonstrated conjugal mechanisms for intergeneric transfer. The availability within food grade lactic acid bacteria of transmissible DNA which encodes industrially significant traits, and the existence of mechanisms for interspecific and intergeneric transfer, indicate that conjugation represents a valuable tool for biotechnological improvement of lactic acid bacteria.

Protoplast fusion. A second biotechnological technique which merits further investigation is protoplast

fusion. This method is based upon observations that the microbial or plant cell wall may be enzymatically removed in hypertonic solution, to yield a plasma membrane bound protoplast, and that a new wall may be regenerated on an appropriate medium (2). Protoplast fusion was originally developed in plant systems by Kao and Michayluk (55), who found that polyethylene glycol (PEG) facilitated fusion between different cells. Regeneration of the fusants produced hybrid cells with characteristics from both parental cell types. Subsequent work with bacteria demonstrated that PEG also induced fusion of these protoplasts (28,111).

Gasson (34) was the first to apply this technology to lactic acid bacteria and demonstrated recombination of both plasmid and chromosomally encoded genes among derivatives of Lactococcus lactis subsp. lactis 712. Okamoto et al. (95) also reported recombination of chromosomal genes among auxotrophic mutants of Lactococcus lactis subsp. lactis as a consequence of protoplast fusion and regeneration. Intergeneric transfer of plasmid and chromosomal genes to lactic acid bacteria has also been demonstrated with this technique (15,133). These results indicate that protoplast fusion may be a powerful tool for the construction of hybrid microorganisms. With this technology, investigators could potentially combine the desirable traits (e.g., flavor, acid, and bacteriocin production) from distinct

genera into one new lactic organism. Such hybrid bacteria could be used both to prepare improved versions of traditional fermented dairy products (i.e., greater shelf stability, improved flavor and texture qualities), and to develop new products based upon the unique metabolic capabilities these organisms might possess. Protoplast fusion may also be an effective method to obtain mutants with increased expression levels of important proteins. Soviet investigators have obtained fusants which expressed a 10-12 fold increase in nisin production levels when compared to the original parental strains (123).

Despite the clear potential that protoplast fusion holds as a powerful tool for the biotechnology of lactic acid bacteria, relatively little investigative attention has been given to the procedure in recent years. Some of this negligence may stem from the need to pre-establish protoplast formation and regeneration conditions for each organism under study (2). Further studies with regard to protoplast formation and regeneration among lactic genera other than lactococci are required if protoplast fusion is to approach the potential it offers for strain construction and improvement.

Electroporation. The development of recombinant DNA technology within the past 25 years has provided modern microbiologists with extraordinary power to precisely alter physiological characteristics of the lactic acid bacteria

used to ferment dairy products. The possibilities for substantial and precise strain improvements have never been greater. In order to apply this technology and genetically alter strains for industrial application, reliable and efficient methods for bacterial transformation must be available (87). The most promising of these methods to emerge in recent years has been electroporation.

Initially developed as a method to facilitate cell fusion in eukaryotes (98), electroporation is a physical treatment based upon the phenomenon of "electric pore formation" in cells (124). Cellular membranes exposed to a high electric field become polarized and develop a voltage potential across the membrane. If this potential exceeds a threshold limit, localized breakdown of the membrane occurs and the cell becomes permeable to extraneous molecules (60,61). Under conditions which must be experimentally established, the breakdown is reversible and treated cells may be recovered. The transfection of mouse fibroblasts by Neumann et al. (92) was the first reported use of electroporation for introduction of exogenous DNA into cells. Although the actual mechanism for DNA entry into cells has remained mysterious, use of the technique has spread to include transfection of plant protoplasts and efficient, high frequency electro-transformation of a variety of bacterial genera and species (113).

Harlander (44) first reported electro-transformation of intact (non-protoplasted) cells of Lactococcus lactis subsp. lactis. The transformation frequencies obtained were comparable to those offered by more difficult and time consuming protoplast transformation procedures (63,89) which previously were the only available means to transform lactic acid bacteria. At approximately the same time, Chassy and Flickinger (10) reported successful and efficient electro-transformation of Lactobacillus casei subsp. casei. Within one year of these reports, the number of successfully electro-transformed species of dairy lactic acid bacteria had grown to include Streptococcus salivarius subsp. thermophilus (119), Lactococcus lactis subsp. cremoris (99,134), Lactobacillus acidophilus, Leuconostoc mesenteroides subsp. cremoris and subsp. dextranicum (75). Perhaps the most encouraging aspect of these reports was the common observation that a single protocol for electroporation allowed transformation of different strains and even different genera of bacteria (10,75,99). This was in sharp contrast to protoplast transformation techniques where investigators found that a given procedure often worked with only a limited number of related strains (75,142,144).

While the list of lactic acid bacteria which have been genetically transformed by electroporation continues to grow (47,143,146), interest has shifted toward

identification of parameters which yield very high transformation frequencies. The capability to efficiently transform cells is directly tied to the ease with which recombinant DNA technology may be applied to a particular bacterium. Among the lactococci, several studies have demonstrated that very efficient transformation frequencies (up to 10^7 transformants/ug DNA) may be obtained if the thick gram-positive cell wall is weakened prior to electroporation (50,99,134). These results suggest that the lactococcal murein layer may act as a barrier to DNA entry but it is unclear whether the same is true of other lactic organisms. Wycoff et al. (143) have obtained high frequency electro-transformation (>10^6 transformants/ug DNA) with whole cells of Leuconostoc mesenteroides subsp. cremoris 44-4. Although significant progress has been realized toward the development of very efficient electro-transformation procedures of lactic acid bacteria, most notably among lactococci, transformation frequencies remain orders of magnitude lower than the 10^{10} transformants/ug DNA reported for electroporation of Escherichia coli (23). Further investigation of the various parameters which affect the efficiency of electro-transformation of lactic organisms may eventually yield results comparable to those for E. coli.

Although most reports of high efficiency electro-transformation in bacteria have involved relatively small

plasmids (23,50,143), the technique has also proven useful to transform larger plasmid DNAs. In work performed at Utah State University, Gillies and Kondo (unpublished data) electro-transformed a Lac$^-$Prt$^-$ strain of L. lactis subsp. lactis, albeit at low frequency, with 55 kb lac and 43 kb prtA plasmids isolated from L. lactis subsp. lactis C20. The relationship between plasmid size and the efficiency of electroporation remains unclear. Some reports have indicated that greater plasmid size adversely effected transformation frequency (75,119) while others have found no obvious relationship between size and electro-transformation efficiency (78,99). Resolution of this question requires direct comparison, as yet not performed, between the transformation frequency of plasmids which contain the same origin, regulatory sequences and markers, and which differ only in size (113).

In conclusion, electroporation presents the most direct and efficient method for the development of a genetic transformation protocol among lactic acid bacteria. The technique offers significant advantages over previous methods and this feature, combined with the commercial availability of reliable instruments, have made electroporation the method of choice for the introduction of exogenous DNA into lactic organisms. For more detailed discussions of the experimental parameters which affect electro-transformation efficiency and cell recovery, or the

events surrounding cell membrane breakdown, see references 113 and 124.

Gene delivery systems. With the development of transformation systems for lactic acid bacteria came a need to construct useful vectors for gene delivery. Cloning vectors may be loosely divided into two general categories; those for experimental research and food grade vectors designed for safe application in food systems. The former typically encode resistance genes to one or more clinically useful antibiotics while the latter must not contain any such DNA. The minimal requirements of either type of vector are that it 1) replicate within the host species of bacteria, 2) encode a gene which facilitates selection of transformed from non-transformed cells, 3) possess unique restriction endonuclease site(s) where DNA fragments may be inserted without damage to replication or selection functions, and 4) be of relatively small size so that recombinant constructs may be readily transformed into host cells. Modern cloning vectors typically include more sophisticated features such as two selective markers and a multiple cloning site located such that insertion of cloned DNA fragments inactivates one of the markers. Loss of the corresponding phenotype is then used to directly select cells with recombinant molecules from those which contain only vector DNA.

The first cloning experiment performed with lactic acid bacteria, reported by Kondo and McKay (63) shortly after they had developed protoplast transformation in lactococci, utilized a vector initially developed for Streptococcus sanguis research. With the advent of transformation, other investigators quickly constructed vectors based upon cryptic lactococcal plasmids (37,62). These vectors proved especially useful because of their ability to replicate in Bacillus subtilus and E. coli hosts, where DNA manipulation techniques were well established. Although this group of vectors remain useful for cloning, expression of heterologous DNA in lactic acid bacteria has required functional lactic expression signals within the cloned DNA fragment.

The next family of laboratory research vectors to emerge were designed to investigate this shortcoming. This group included vectors developed to collect lactic promoter and terminator sequences (1,19,117,118,136), and protein secretion signals (96,114,116). These vectors have been utilized to obtain important information on gene expression which has in turn provided the basis for sophisticated expression and secretion vectors (115-117,132). The latter two types of cloning vectors specifically promote expression and secretion in lactic organisms of protein from cloned, heterologous DNA. The development of expression and secretion vectors for lactic acid bacteria

has been an important advancement because these tools are prerequisites for these organisms to be utilized in the manufacture of genetically engineered proteins.

The final class of experimental gene delivery systems under development for lactic acid bacteria are integration vectors (70,97,102). In contrast to the vectors described above, which typically exist in host cells as extrachromosomal plasmid molecules, integration vectors are designed to recombine with the host chromosome upon cell entry. Interest in construction of these vectors has stemmed from the inherent instability of plasmid DNA in cells which can produce the concomitant and irreversible loss of any traits they encode. It was the instability of several important metabolic traits in lactococci that first led investigators to examine these organisms for plasmid DNA (86). Since then the genes which encode a number of these traits have indeed been located upon various plasmid DNAs (79). One potential method to stabilize important genes in lactic acid bacteria is through chromosomal integration. As discussed previously, McKay and Baldwin (81) were the first to demonstrate stabilization of plasmid genes in a lactic organism. They employed transduction to integrate genes for lactose utilization and proteinase into the chromosome of a L. lactis subsp. lactis C2 derivative and demonstrated improved stability of these traits in the construct.

Modern integration vectors are designed to achieve comparable results with any gene of interest. Most of the integration vectors developed for lactic acid bacteria utilize host mechanisms for homologous DNA recombination to enter the chromosome (70,97). Raya et al. (102), however, have constructed an integration vector for Lactobacillus gasseri which instead utilizes integrase and attP functions isolated from the genome of a temperate bacteriophage. Although a relatively new development in lactic acid bacteria, Leenhouts et al. (69) have already demonstrated stabilization of proteinase genes in Lactococcus lactis subsp. lactis with an integration vector.

Information gleaned from the work outlined above has provided investigators with a solid framework for gene cloning and expression in lactic acid bacteria. This background information was also necessary for the development of successful food grade gene delivery systems. In principle, food grade vectors are identical to their laboratory counterparts. Because they may become distributed in foods, however, safety concerns dictate that food grade vectors cannot employ antibiotic resistance genes as selective markers. The concern is justified because these genes may be transmitted inadvertently to pathogenic microorganisms (83). Because of this concern, all of the potential food grade systems constructed to date have utilized selective markers isolated from food grade,

generally regarded as safe (GRAS) bacteria. The lactic markers employed in these vectors include genes for nisin resistance (32,137,140), β-galactosidase (125), or thymidylate synthase (103). A number of other lactic genes, such as those which encode bacteriocin production and immunity or the utilization of various carbohydrates, may also be useful selective markers for food grade gene delivery systems.

Although reports which describe potential food grade vectors have emerged only recently, it is probable that more sophisticated versions will follow quickly. Leenhouts et al. (71) recently discussed a method to construct a potential food grade integration vector. Before any of these food grade vectors may be utilized to improve bacteria utilized in food, the constructs will have to obtain regulatory clearance. At present no precedent for such a food application exists and the regulatory process may ultimately determine the role of these vectors for biotechnology in fermented foods.

Regulatory Aspects of Dairy Biotechnology.

Substantial progress has now been made toward the isolation of genes important to dairy fermentations and the food grade delivery systems needed to introduce those genes into the fermentative microorganisms. Before these systems may be commercially applied to foods, the safety of new organisms and products must be verified and approved by the

agencies which regulate food safety in each country. Within the United States, those agencies include the Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA).

Because the regulation of biotechnology involves several United States government agencies, the Executive Office of the President, Office of Science and Technology, established the Biotechnology Science Coordination Committee (BSCC). The role of the BSCC is to develop a unified federal policy which will avoid potential conflicts between the various agencies. The committee has designed a coordinated framework for the regulation of biotechnology which indicates that new products derived from biotechnology should be reviewed by the respective agencies in essentially the same manner as products manufactured under more traditional processes (25). With respect to foods and food products obtained from biotechnology, this means jurisdiction rests with FDA and USDA.

Commercial manufacturers of dairy foods are subject to regulations from both FDA and USDA; FDA promulgates standards of identity which describe and define characteristics of individual dairy products, while USDA regulates grading of finished dairy products (67). Because actual production of these foods is regulated by FDA, organisms or products derived from biotechnology for dairy foods applications should require approval from FDA only

prior to use in food.

Although FDA issued a policy statement for regulation of biotechnology products in 1986 (29), important questions exist with regard to how the agency will view genetically altered microorganisms in human food. For example, would a recombinant DNA molecule derived entirely from the DNA of generally regarded as safe (GRAS) organisms, and subsequently transformed into another GRAS bacteria, yield an organism which would require approval as a food additive? What about transformation of a native plasmid isolated from one GRAS bacteria into another? In the latter situation, would approval require, as dictated for recombinant molecules, that the entire DNA sequence of the plasmid be provided to FDA? How will FDA view intraspecific versus interspecific and intergeneric constructs, achieved by duplicate technology, of GRAS microorganisms? At present, FDA policy toward biotechnology has focused upon case by case review and approval (29). Biotechnology is an expensive process, however, and until these issues are clarified and a firm prospect for regulatory approval of genetically altered microorganisms exists, it is unlikely that the dairy industry will embrace this new science. Unfortunately, events in recent years have not clarified the FDA position toward the use of genetically engineered microbes in human food.

The only connected development in recent years entailed the commercial utilization of lactococci with conjugally improved bacteriophage resistance (109). FDA has not interfered with this application and did not require any safety review of these microorganisms. This stance indicated that FDA believes conjugation among GRAS bacteria does not increase the danger these organisms pose to human health and safety. This is an important precedent because, as discussed earlier, conjugation may be quite useful for the genetic improvement of lactic acid bacteria. Further insight into the FDA position may be available from proposed USDA guidelines which pertain to the release of genetically altered organisms into the environment (131). The validity of such an inference is based upon the actions of BSCC (25), whose mission is to ensure consistent and coordinate biotechnology regulations among federal agencies, and because altered microorganisms in food will likely become distributed into the environment. Among other things, the USDA document specifically proposes to exclude from regulation microorganisms modified solely by movement of nucleic acids, if they have not first been manipulated <u>in vitro</u>, through physiological processes such as conjugation, transduction, and transformation.

While transduction and conjugation are documented physiological processes among lactic acid bacteria (26), natural transformation systems in these organisms have not

been identified. As discussed above, the latter form of gene transfer is today usually achieved by electroporation; a technique based upon physical phenomena rather than natural physiology. The conclusions drawn from this inference are that the FDA position toward the safety of organisms which are genetically altered by physiological processes will likely be determined by the status of the parental organisms. The agencies opinion of bacteria improved by transformation, however, remains unknown, even if recombinant DNA molecules have not been employed in the construct. The latter condition provides an important distinction because two situations may be envisioned with regard to the safety review of lactic organisms genetically altered by transformation. The first involves transformation into one GRAS organism of whole, unaltered native plasmids isolated from another GRAS bacteria, while the second applies to recombinant DNA molecules.

One of the most significant discoveries to emerge from studies of lactic acid bacteria, particularly lactococci, has been that many important traits for milk fermentations are encoded by plasmid DNA (79). If some of these plasmids were transformed into industrial strains, they would probably contribute an immediate refinement to the fermentation. The interests of the dairy industry would certainly be served if the question of how intraspecific and intergeneric constructs of transformed GRAS bacteria,

which employed whole, unaltered plasmids from other GRAS bacteria, will be addressed by FDA. These answers may require the dairy or culture industry to submit test cases to FDA, the expense of which cannot be foreseen.

The criteria for FDA review of organisms obtained through the use of recombinant DNA has been established (29). It remains unclear whether the agency would consider a transformed GRAS organism, which contained a recombinant DNA molecule derived entirely from other GRAS bacteria, as GRAS or as a food additive. FDA has clearly indicated that the latter situation is possible but has stated it will review petitions case by case (29). Food additive approval requires considerable time and expense, features which industry chooses to avoid whenever possible. Furthermore, petitions for GRAS affirmation of new products and organisms require the same scientific data as those for food additives plus documentation of literature which supports GRAS designation (27). Thus, GRAS affirmation may be even more laborious to a company than approval as an additive. Because of the potential expense, clarification of the FDA position is critical if biotechnology is to be accepted by the dairy industry. Resolution of this issue will require action by the dairy fermentation industry, either through dialogue or submission of test cases. Even greater confusion surrounds the status of hybrid microorganisms which might be obtained by protoplast fusion

technology. It appears that at present these organisms, because they may involve substantial yet poorly defined DNA recombination, would receive added scrutiny during review and are the most probable constructs to receive food additive status. In summation, although progress has been achieved further action is required of both dairy foods producers and FDA to clarify impressions regarding the safety of genetically altered microorganisms in fermented foods.

While clear guidelines based upon scientific fact will serve to promote the application of biotechnology in the dairy foods industry, the success of these products will also rely heavily upon public knowledge of biotechnology. As discussed recently by Harlander (45,46), consumer perceptions and fears toward food biotechnology cannot be trivialized or ignored if the technology is to succeed. A good example of the dangers which face new developments in biotechnology is offered by the global controversy which has surrounded the proposed use of recombinant DNA-derived bovine somatotropin (r-bST) to increase milk production in dairy cattle (16,77,104). Although FDA concluded several years ago that milk obtained from cattle treated with this hormone was safe for human consumption, and use of r-bST will likely be approved by FDA before the end of 1991 (104), negative consumer perceptions could determine the eventual success of this biotechnological development

(77,104). Further obstacles to the application of biotechnology may result from state or local regulations. Legislation at this level is far more susceptible to amendments which address public concerns and yet lack scientific basis, than is federal law.

In summary, biotechnology holds great potential to improve and expand our supply of fermented dairy foods. If this potential is to be realized, both industry and consumers must accept the new science. Acceptance of biotechnology among the dairy fermentation industry may be accelerated by clarification of the FDA perspective toward the use of genetically altered microorganisms in foods. Acceptance among the general public will require education and dialogue. Harlander (46) has provided an excellent outline designed to meet the challenges posed by public misconceptions of biotechnology. If these challenges are met, the dairy industry could experience an unprecedented revolution in product quality, variety, and supply all as a consequence of biotechnology.

Nisin. The bacteria utilized to produce fermented dairy foods produce a number of organic compounds which are antagonistic to other microorganisms. Combined, these products help to create an environment within the fermented food that strongly inhibits the growth of pathogenic and spoilage microorganisms. Examples of these antimicrobial compounds include organic acids such as lactate, acetate

and propionate, and other compounds such as ethanol, hydrogen peroxide, and proteinaceous bacteriocins. The unique physical and inhibitory properties of the latter compounds has generated considerable interest toward their application as food preservatives.

Bacteriocins have been found among both gram-positive and gram-negative species and, in general, these molecules exert a bactericidal effect only toward closely related species of bacteria. Some of the bacteriocins produced by gram-positive bacteria, which include microorganisms used for dairy fermentations, exhibit a much broader spectrum of antagonism. These antimicrobial molecules may act not only against related species but also against unrelated, pathogenic and spoilage bacteria and even fungi. Bacteriocin production has been demonstrated in nearly every genera of lactic acid bacteria (58) as well as within the propionibacteria (76). Because of their proteinaceous nature, bacteriocins are degraded by stomach enzymes when consumed as part of a fermented food. This feature combined with useful physical and inhibitory properties has prompted the widespread utilization of one of these compounds, nisin, as a preservative for processed dairy and vegetable foods (Table 1).

Nisin is a bacteriocin produced by some strains of Lactococcus lactis subsp. lactis (formerly called Streptococcus lactis). This organism is used in the

TABLE 1. Examples of current and potential applications for nisin

Food applications[a]:

Dairy products:
- -cheese
- -processed cheese, cheese spread, and cheese food
- -cheese powder
- -pasteurized milk
- -flavored milks
- -evaporated milk
- -nonrefrigerated milk
- -buttermilk
- -confectionery and clotted cream
- -desserts
- -yogurt

Canned foods:
- -vegetables
- -soups
- -tomato paste and puree
- -mushrooms

Other foods:
- -alcoholic beverages
- -bakery products and fillings
- -margarine
- -mayonnaise
- -meats

Medicinal applications:

Animal:
- -prevention and control of bovine mastitis.

Human:
- -mouthwash
- -prevention and control of acne.

Other applications[a]:

- -ice for storing fresh fish.
- -prevention and control of gram-positive contamination in industrial fermentations which utilize gram-negatives, yeasts, or fungi.
- -improved silage quality

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[a]Portions adapted from Delves-Broughton (17).

manufacture of cheeses, such as Cheddar, that require mesophilic starter cultures. Nisin inhibits a variety of gram-positive bacteria (52,73), and some gram-negatives may also be inhibited (120). Investigations into the mechanism of inhibition have shown that nisin forms pores in the cytoplasmic membrane of susceptible bacteria and produces rapid membrane depolarization (65,66,108). Exposure to nisin also induced autolysis in Staphylococcus simulans (6). Other work has shown the bactericidal action of nisin is dependent upon the unusual dehydro residues in the protein (43), which react with sulfhydryl groups in the cellular target (42,74,90).

Often inappropriately described as an antibiotic, nisin is unique by virtue of its global acceptance as a food preservative (17,52). This application is possible because unlike true antibiotics such as penicillin and tetracycline, nisin is degraded by stomach proteases when eaten and nisin-resistant organisms in the gut do not exhibit cross-resistivity to clinically important antibiotics (48,52). In Europe, nisin has enjoyed over 30 years of successful application as a preservative in the food industry and, in 1988, the Food and Drug Administration approved nisin for use in the United States as a direct food additive "to inhibit the outgrowth of Clostridium botulinum spores and toxin formation in certain pasteurized cheese spreads" (30).

In addition to the proven applications for nisin as an added preservative in processed foods, studies have suggested that benefits may also be obtained from the use of nisin-producing starter cultures in fermented dairy products. European investigators used nisin-producing starter cultures, alone or in conjunction with their usual commercial starters, to control clostridial spoilage in rennet-set Dutch and Swiss cheeses (Edam, Emmental). Unfortunately, these investigators also found that while the nisin-producing starter controlled clostridial fermentations, defects in cheese quality usually resulted. These defects were attributed to 2 factors: 1) the usual commercial cultures, which produced quality cheese, were all nisin sensitive, and 2) the L. lactis subsp. lactis nisin-producers did not possess the traits required to produce quality cheese (52,73,127). Subsequent blends of nisin-producers with nisin-resistant variants obtained from the usual commercial cultures also failed because the variants suffered from prolonged lag phase, increased sensitivity to bacteriophages, decreased heat resistance and reduced acid production in milk (73). Although unable to produce a quality cheese with nisin-producing starter cultures, these studies demonstrated that nisin-producing starters inhibited cheese spoilage bacteria and reviewers agreed the work deserved further attention (52,73).

The nisin sensitivity of starter cultures utilized for dairy fermentations has continued to prevent the inclusion of a single nisin-producing strain and has limited the use of nisin to direct addition in processed products. Despite this limitation, nisin-producing dairy starters have remained appealing because, in addition to clostridial species, the bacteriocin inhibits other pathogenic and spoilage organisms such as Listeria and Staphylococcus spp., which are known to contaminate cheeses (5,9,52,73,120).

An outbreak of listeriosis in 1985, which was linked to consumption of a Mexican-style cheese, killed several people in California and the following year, L. monocytogenes was found in Brie cheese imported from France (105). Although this organism does not survive pasteurization, it has been isolated from pasteurized milk, which suggested improper pasteurization or post-pasteurization contamination (5). Since the 1985 outbreak, researchers have shown that L. monocytogenes in contaminated milk will survive the manufacture processes of Camembert, cottage, and Cheddar cheeses (105-107). Benkerroum and Sandine (5) found that although L. monocytogenes grew in cottage cheese at pH 4.9 under refrigeration, the addition of nisin killed the pathogen. Other investigators have also reported inhibition of this important pathogen by nisin (9,120). Nisin inhibition of

Staphylococcus aureus, another pathogen known to contaminate cheeses (4), and other staphylococci has also been demonstrated (73).

In addition to the potential for improved food safety, nisin-producing starter cultures are also appealing for economic reasons. Cheese plants may be able to reduce spoilage losses and cheese processors that utilized cheeses made with nisin-producing starters could decrease the amount of expensive added nisin needed to achieve permissible levels in processed cheese recipes.

The availability of modern gene transfer techniques should permit the construction of nisin-producing dairy starter strains from proven, commercial strains. These constructs would inhibit undesirable spoilage and pathogenic bacteria, yet avoid the problems that were previously associated with the use of nisin producers in dairy fermentations.

Molecular studies of nisin production have demonstrated that nisin is synthesized as a precursor protein that requires post-translational modification to introduce the unusual amino acids lanthionine, dehydrobutyrine, and dehydroalanine, that characterize the mature bacteriocin (43,52). As a consequence, successful introduction of the nisin-producing capability (Nip^+) into dairy organisms requires DNA that encodes not only the nisin precursor (prepronisin) but also genes for the

modifying enzymes and nisin immunity. To this effect, considerable interest has been focused on whether Nip$^+$ is a plasmid or a chromosomally encoded trait.

Possible plasmid involvement has been supported by plasmid curing studies (33,41,68,122) and reports that novel plasmid bands were sometimes observed in some Nip$^+$ transconjugants (35,130). In addition, one team of investigators reports to have cloned the prepronisin gene (*nisA*) from a very large plasmid in *Lactococcus lactis* subsp. *lactis* 6F3 (54). Despite these results, the genetic loci required for nisin-production have not been firmly linked to plasmid DNA and recent evidence has indicated that in several Nip$^+$Suc$^+$ strains, these genes may be encoded by a conjugative transposon located in the lactococcal chromosome (51,101).

From libraries of nisin-producing *Lactococcus lactis* subsp. *lactis* genomic DNA, investigators have cloned and sequenced approximately 3 kb of DNA which contained *nisA* and flanking sequences (8,21,54). Although secretion of pronisin has been detected in *E. coli* (20), production of mature, active nisin by clones has not been reported. Furthermore, lactococcal strains carrying the cloned *nisA* fragment were not immune to nisin (21).

Additional studies have revealed that although nisin activity is not detected in a Nip$^+$ culture until approximately 5 hours of growth, *nisA* is expressed

constitutively (8). This observation has indicated that at least one gene which encodes a maturation enzyme (8) is not expressed with nisA, but is instead regulated under a separate promoter. The locations of the genes which encode nisin immunity and enzyme(s) involved in maturation remain unknown. Furthermore, results of pulsed-field gel electrophoresis (PFGE) experiments have indicated that approximately 70 kb of DNA may be transferred, apparently as a single block of genetic material, from donor to recipient during conjugal transfer of Nip^+ (51). Consequently, the possibility exists that some or all of these loci may be relatively distant from the nisA locus. For this reason, recombinant DNA technology may not be the most practical or direct method to introduce the Nip^+ phenotype into lactic acid bacteria for dairy fermentations.

Another important limitation effecting the use of recombinant DNA methodology are the regulatory barriers that apply to organisms used in the manufacture of food (29). As discussed above, FDA has yet to approve any recombinant DNA-derived organism for use in human food so the expense and difficulty that such organisms will encounter cannot be predicted.

A second approach to develop Nip^+ cheese starters is conjugation. Unlike the former approach, organisms engineered by this method do not receive vector DNA that

may not have come from safe, food-grade bacteria. As a result, strains improved by conjugation are not subject to stringent regulatory review (26,109). This method of gene transfer has proved useful for studies of the genetics and plasmid biology of lactic acid bacteria. Conjugation has also been successfully applied to improve bacteriophage resistance in lactococcal starter cultures for the dairy industry (109). Conjugally improved strains may also experience greater consumer acceptability over strains which contain recombinant DNA. For these reasons conjugation is an important tool for development of genetically improved starters for industrial applications.

Gasson (35) first reported conjugal transfer of Nip^+ among strains of L. lactis subsp. lactis in 1984, and the simultaneous transfer of sucrose-fermenting ability (Suc^+). Gonzales and Kunka (41) later described transfer of the Nip^+Suc^+ phenotype into a strain of L. lactis subsp. lactis biovar. diacetylactis and discovered the transconjugant had also acquired resistance to a lytic bacteriophage. Murphy et al. (91) confirmed reduced bacteriophage sensitivity (Rbs^+) in Suc^+ transconjugants of L. lactis subsp. lactis. Steele and McKay (122) investigated the genetic basis of Nip^+Suc^+, and mentioned conjugal transfer of these genes into an atypical strain of L. lactis subsp. cremoris. As discussed previously, PFGE analysis of genomic DNA from Nip^+Suc^+ transconjugants has demonstrated that

approximately 70 kb of DNA is transferred during conjugation of Nip^+Suc^+ (51) and recent reports have suggested that the gene for N^5-(carboxyethyl) ornithine synthase may also be included in this block of DNA (22,129). In all reports the frequency of Nip^+Suc^+ conjugal transfer has been relatively low, approximately 10^{-7} transconjugants/donor CFU. Despite this limitation, these observations have demonstrated a potential genetic basis for construction of Nip^+ starter cultures that should encounter little regulatory opposition.

In addition to new applications for fermented dairy foods preservation, nisin may also provide useful clinical functions (Table 1). The absence of cross-resistivity to true antibiotics among nisin resistant bacteria (52) indicates that both clinical and food applications may be acceptable for nisin. One potential application involves the treatment or prevention of bovine mastitis caused by gram-positive pathogenic bacteria. The economic losses associated with this affliction are substantial (7) and the threat of residual antibiotics in milk from treated cows has created health and economic concerns among consumers and the dairy industry. Because nisin is most effective against gram-positive bacteria and yet is harmless to humans when eaten (48), the protein may offer a new approach to the control and prevention of bovine mastitis.

Conclusion. The research presented in the next three chapters investigated the hypotheses that nisA was plasmid encoded and whether conjugation could be used to introduce nisin production capability into other lactic acid bacteria. Included within Chapters III and IV are studies into physiological characteristics of nisin-sucrose conjugal transfer in lactococci. Finally, the fourth chapter describes a study into a potential application for nisin as a agent to control bovine mastitis.

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CHAPTER II

LOCATION OF THE NISIN GENES IN THE PRODUCER ORGANISM

ABSTRACT

Plasmid curing, conjugation, and transduction were utilized to investigate whether genes which encode nisin biosynthesis (Nip^+) and sucrose utilization (Suc^+) in Lactococcus lactis subsp. lactis strains 11454 and 7962, were located on plasmid or chromosomal DNA. Although efforts to transduce Nip^+ or Suc^+ were not successful, these traits were detected in derivatives of 7962 which had been cured of all detectable plasmid DNA, and in 11454 derivatives which had been cured of the 29.1 Megadalton plasmid previously linked to Nip^+Suc^+. Moreover, agarose gel electrophoresis failed to reveal the presence of novel plasmid molecules in lysates of Nip^+Suc^+ transconjugants. Finally, DNA-DNA hybridizations were performed between plasmid and genomic DNA isolated from several nisin-producers and an oligonucleotide probe which detected the nisin structural gene nisA. The results confirmed that nisA was not associated with detectable plasmid DNA in any of the nisin producers examined but was present in chromosomal DNA preparations.

INTRODUCTION

Nisin is a 34 amino acid bacteriocin synthesized by some strains of Lactococcus lactis subsp. lactis (15). For over 30 years, European food processors have employed nisin as a preservative for processed vegetable and dairy products (6,15). Within the United States, the Food and Drug Administration recently approved nisin as an antibotulinal additive for pasteurized cheese spreads (9). These applications have been possible because nisin is an effective inhibitor of gram-positive pathogenic and spoilage bacteria, including spore-forming clostridia and bacilli, and because the protein is readily degraded by stomach enzymes when consumed (13,15). Because of the economic importance nisin holds in the food industry, considerable interest has focused upon the genetics of nisin biosynthesis.

Evidence for plasmid involvement in nisin biosynthesis (Nip^+) was first offered by Kozak et al. (19) whom noted that plasmid curing conditions stimulated the frequency of nisin-negative (Nip^-) mutants. This observation was quickly followed by a report which indicated that some Nip^- mutants had also lost plasmid DNA (10). LeBlanc et al. (20,21) noted that Nip^- mutants of L. lactis subsp. lactis 11454 had lost a plasmid of approximately 29 Megadaltons (MDa) and the ability to utilize sucrose (Suc^-). Sucrose

utilization (Suc^+) is uncommon among lactococci and a relationship between Nip^+ and Suc^+ had previously been noted (15). Two subsequent investigations supported the observation of LeBlanc et al. (20) which indicated that the 29 MDa plasmid of 11454 was linked to Nip^+Suc^+ (12,37). Genetic evidence for linkage of Nip^+Suc^+ was provided when Gasson (11) discovered that these two phenotypes were conjugally cotransferred. Additional evidence for plasmid involvement came from reports that novel plasmid molecules were detected in Nip^+Suc^+ transconjugants (11,45) and that this phenotype could be transferred to a recombination deficient (Rec^-) recipient (37). Furthermore, several laboratories have now cloned the nisin structural gene, nisA (5,7,16), and one report indicated that the gene was isolated from a very large plasmid in L. lactis subsp. lactis 6F3 (16). Despite these results, the genetic loci required for nisin biosynthesis have not been firmly linked to plasmid DNA and recent evidence has indicated that in several strains, Nip^+ is encoded by a conjugative transposon located in the lactococcal chromosome (14,33).

Identification of a plasmid which encoded Nip^+ would facilitate studies of nisin biosynthesis and may allow rapid development of nisin-producing dairy starter cultures through transformation. For these reasons this laboratory decided to investigate the nisA locus in strains where Nip^+ had been firmly linked to plasmid DNA. L. lactis subsp.

lactis 7962 and 11454 were two well characterized strains in which strong evidence for plasmid involvement had been reported (12,20,37,45).

This report describes the use of plasmid curing, conjugation, transduction, and DNA-DNA hybridizations to locate genes for Nip^+Suc^+ in 7962, 11454 and other nisin-producing strains. Although efforts to transduce these phenotypes proved unsuccessful, curing studies demonstrated that plasmid loss did not affect nisin production in derivatives of strain 7962 or 11454. Furthermore, plasmid analysis of lysates from Nip^+Suc^+ transconjugants did not reveal novel plasmid molecules in recipient backgrounds. Finally, DNA-DNA hybridizations to plasmid and chromosomal DNA isolated from several nisin-producers were performed with an oligonucleotide probe which detected nisA. Results indicated that nisA was located in the chromosome of Nip^+Suc^+ L. lactis subsp. lactis strains 7962, 11454, DL16, 496, and was not associated with detectable plasmid DNA in any of the nisin producers examined.

MATERIALS AND METHODS

Bacterial Strains. Lactococcal strains utilized in this study are listed in Table 1. Lactococci were propagated at 30°C in M17 broth (41) which contained 0.5% glucose or lactose (M17-G or M17-L) as the sole carbohydrate source. Escherichia coli strains V517 (24) and MH613 (35) were grown in Brain-Heart Infusion (BBL

TABLE 1. *Lactococcus lactis* subsp. *lactis* strains used in the study

Strain	Relevant phenotype[a]	Description (reference)
ATCC 11454	$Nip^+Suc^+Em^sStr^s$	Nip^+Suc^+ donor (37).
ATCC 7962	$Nip^+Suc^+Em^sStr^s$	Nip^+Suc^+ donor (45).
DL16	$Nip^+Suc^+Em^sStr^s$	Nip^+Suc^+ donor (37).
496	$Nip^+Suc^+Em^sStr^s$	Nip^+Suc^+ donor obtained from the National Dairy Research Institute, Karnal, India.
LM0230	$Nip^-Suc^-Str^sEm^s$	Plasmid-cured recipient derived from strain C2 (8).
LM2301	$Nip^-Suc^-Str^r$	Plasmid-cured recipient derived from strain C2 (47).
LM2302	$Nip^-Suc^-Em^rStr^r$	Plasmid-cured recipient derived from strain C2 (47).
LM2306	$Nip^-Suc^-Em^rStr^r$	Plasmid-cured recipient derived from strain C2 (37).
JBR13	$Nip^-Suc^-Rec^-Em^rStr^r$	Recombination deficient strain MMS367 (38) electro-transformed with pGK-13 (40, this study).
JB0213	$Nip^-Suc^-Em^r$	LM0230 electro-transformed with pGK13 (40, this study).
KG491	$Nip^-Suc^-Em^r$	C20 (28) derivative which contained only the 48 kb parental plasmid, electro-transformed with pGK13 (40, this laboratory).
JK2301β	$Nip^-Suc^-Em^r$	LM2301 transconjugant (18) which contains pAMβ1 (22).
NS5406	$Nip^+Suc^+Em^r$	Nip^+Suc^+ transconjugant from 11454 x LM2306 (4).

[a]Abbreviations: Nip^+, nisin production and immunity; Suc^+, sucrose utilization; Em^r, erythromycin resistant; Str^r, streptomycin resistant; Rec^-, recombination deficient.

Microbiology Systems, Cockeysville, Md.) at 37°C with aeration. Bacterial cultures were stored at 4°C and maintained by biweekly transfers.

Plasmid curing. To obtain derivatives of strains 11454 and 7962 which had lost plasmid DNA, these cells were propagated either at 37°C (19,29) or in the presence of 10 ug per ml of the DNA gyrase inhibitor novobiocin (26). After three serial transfers under curing conditions, cells were diluted in 0.85% saline and 100 ul aliquots were plated upon bromocresol purple (BCP)-lactose indicator agar (29). Colonies which exhibited impaired acid production were purified and subjected to plasmid analysis.

Transduction. Induction of prophage from lactococcal nisin-producers was performed with mitomycin C or ultra-violet radiation as described by Park and McKay (32). Phage preparations were concentrated and purified by polyethylene-glycol (PEG) sedimentation and $CsCl_2$ density gradient centrifugation as described by Klaenhammer and McKay (17). Transductions were performed by the method of McKay et al. (30).

Conjugal matings. Conjugations were performed by the direct-plate method previously developed in this laboratory (4). Transconjugants were verified by phenotypic characterization and plasmid analysis. Because the purpose of this study was only to investigate plasmid involvement in Nip^+Suc^+, conjugal transfer frequencies were not

included in this report.

DNA isolation and purification. Plasmids were isolated from lactococci by the method of Anderson and McKay (1), and from E. coli strains by the alkali-sodium dodecyl sulfate lysis procedure of Maniatis et al. (25). If required, plasmid DNA was purified by centrifugation through $CsCl_2$ density gradients (25). The presence of plasmids in cell lysates was established by electrophoresis in 0.6% agarose gels at 3 V/cm for 7 h with $CsCl_2$-purified plasmids from E. coli V517 (24) included for plasmid size standards.

Lactococcal genomic DNA was isolated by a previously described modification (4) of the Anderson and McKay procedure (1). Restriction analysis of genomic DNA was performed as per the enzyme manufacturer's directions (Bethesda Research Laboratories, Gaithersburg, Md. or International Biotechnologies Inc., New Haven, Conn.). Prior to electrophoresis, 3 ul of RNase A (Sigma Chemical Co., St. Louis, Mo.; 1 mg/ml in 10 mM Tris-HCl, pH 8.0) was added to each sample. Restricted genomic DNA was separated in 0.8% agarose gels at 1.4 V/cm for 18 h. Adenovirus type 2 DNA, digested with EcoRI and BamHI (International Biotechnologies Inc.), or the American Synthesis Inc. (Pleasanton, Calif.) Raoul™ I marker, were utilized for fragment size standards. When required, DNA fragments were purified from agarose gels with a USBioclean kit (United

States Biochemical, Cleveland, Ohio).

Gene probes and DNA-DNA hybridizations. To detect the nisin structural gene, nisA, a 14-mer oligonucleotide probe (5'-ATGTTACAACCCAT-3') was synthesized and purified as described previously (4). Plasmid pMH613 (35) was utilized to probe lactococcal plasmid and genomic DNA for sucrose genes. This molecule contained Streptococcus mutans genes for the sucrose-specific permease, enzyme-II^{scr} (scrA), and sucrose-6-phosphate hydrolase (scrB), which are required for sucrose utilization by a phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS).

Transfer of DNA to GeneScreenPlus™ nylon membranes (E.I. du Pont de Nemours & Co., Inc., NEN Products, Boston, Mass.) was performed by the method of Southern (36). The oligonucleotide nisA probe was 5'-end-labelled with [γ-^{32}P]ATP and then hybridized to plasmid and genomic DNA in NaCl-sodium citrate solution as described by Ausubel et al. (3). Sucrose gene fragments isolated from pMH613 were also 5'-end-labelled by this technique. Incorporation of [α-^{32}P]CTP into pMH613 was performed with a New England Nuclear (E.I. du Pont de Nemours & Co., Inc., NEN Products) NEK-004 nick translation kit. Hybridization of pMH613 and sucrose gene fragments to plasmid and chromosomal DNA in NaCl-sodium citrate solution was performed as described by Maniatis et al. (25). Autoradiography was performed as described by Ausubel et al. (3).

Assays. Nisin production was verified by agar overlay as described by Steele and McKay (37). The ability to ferment sucrose was determined by culture growth and acidification upon BCP-sucrose agar.

RESULTS

Plasmid curing. The effect of plasmid loss upon nisin production in L. lactis subsp. lactis strains 7962 and 11454 was investigated. Plasmid analysis of 7962 isolates, obtained after serial transfers in M17-G broth which contained 10 ug of novobiocin per ml, identified derivatives which possessed only one or none of the parental plasmids (Fig. 1). When agar overlay assays for nisin production were performed with these derivatives, all had retained this capability (Table 2). Plasmid analysis of 11454 isolates collected after curing treatments identified two derivatives, JB1100 and JB1101, which no longer contained the 29.1 MDa parental plasmid (Fig. 2). This plasmid had been linked to the Nip^+Suc^+ phenotype by several investigators (12,20,37). Assays performed in this study, however, demonstrated that 11454 derivatives cured of the 29.1 MDa plasmid expressed Nip^+Suc^+ (Table 2). Loss of lactose-fermenting ability (Lac^+) was noted in derivative JB1101, which was cured of the 31.8 MDa parental plasmid (Fig. 2). Transformation had previously been utilized to demonstrate that Lac^+ was encoded by this molecule (37).

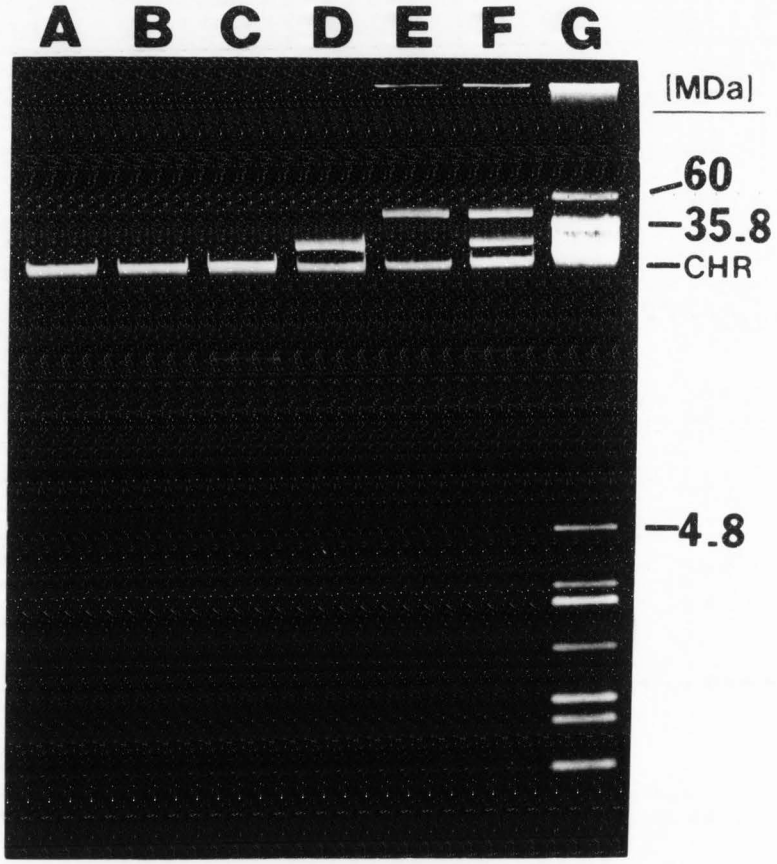

FIG. 1. Agarose gel electrophoresis of plasmid DNA isolated from L. lactis subsp. lactis 7962 and plasmid-cured derivatives. The DNA samples were isolated from; A and B) plasmid-cured derivatives JB704 and JB705, C) JB707, which retained only the 13 MDa parental plasmid, D) JB703, which possessed the 30 MDa parental plasmid, E) JB702, which contained only the 45 MDa parental plasmid, F) L. lactis subsp. lactis 7962, with plasmids of 13, 30 and 45 MDa, and G) E. coli V517 size standard plasmids (24). Size estimates of 7962 plasmid DNA were from Orberg (31).

TABLE 2. Nisin production and sucrose utilization in plasmid-cured derivatives of L. lactis subsp. lactis 7962 and 11454

Strain	Plasmids (MDa)	Nip[a]	Suc[b]
L. lactis subsp. lactis:			
ATCC 7962	45, 30, 13[c]	+	+
JB702	45	+	+
JB703	30	+	+
JB707	13	+	+
JB704 and JB705	cured	+	+
ATCC 11454	31.8, 29.1, 21.9, 19.4, 3.6, 1.49[d]	+	+
JB1100	31.8, 21.9, 19.4, 3.6, 1.49	+	+
JB1101	21.9, 19.4, 3.6, 1.49	+	+

[a]Nisin production as determined by the agar overlay method of Steele and McKay (37), + = positive result.
[b]Able to ferment sucrose as determined by acidification of BCP-sucrose agar, + = positive result.
[c]Size estimates from Orberg (31).
[d]Size estimates from LeBlanc et al. (21).

Transduction. L. lactis subsp. lactis strains 7962, 11454, and DL16 were treated with UV radiation or mitomycin C to induce prophage (32). None of these Nip^+Suc^+ strains exhibited a decrease in A_{600} within 6 h after induction,which normally would indicate cell lysis with phage release (32). Nevertheless, supernatant was collected from each strain 6 h after treatment and subjected to PEG sedimentation and $CsCl_2$ density gradient centrifugation to concentrate and purify phage which might be present (17). After centrifugation, 7962 $CsCl_2$ density gradient tubes exhibited three distinct blue-colored bands, while 11454 and DL16 tubes each yielded one band (data not shown). The putative phage bands (17) were collected and

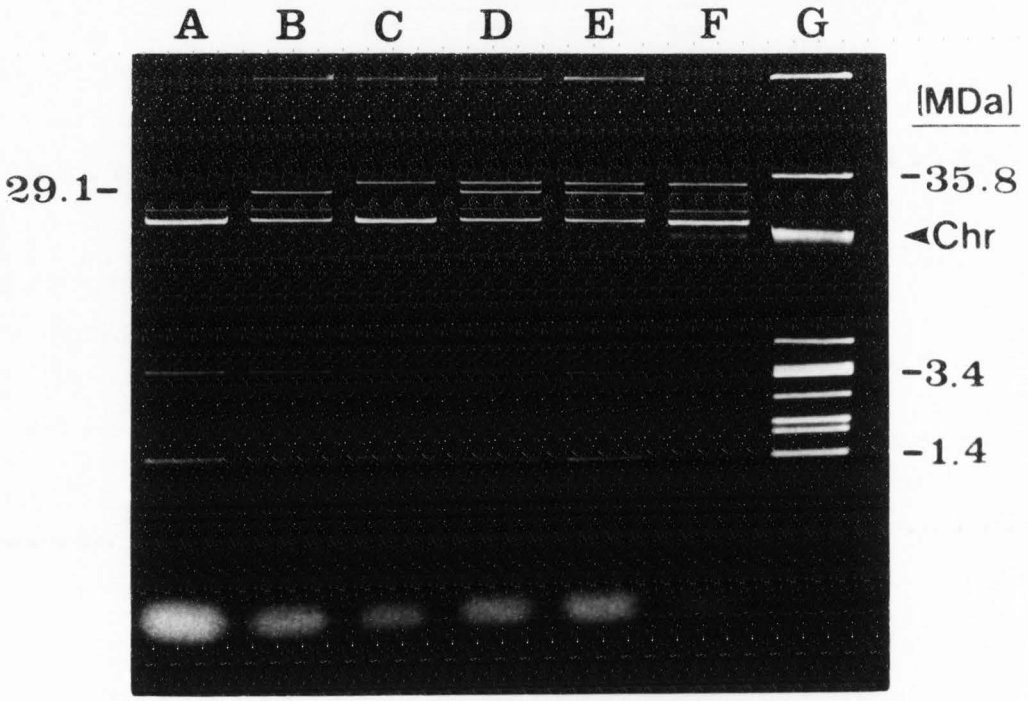

FIG. 2. Agarose gel electrophoresis of plasmid DNA isolated from L. lactis subsp. lactis 11454 and plasmid-cured derivatives. The gel contained DNA isolated from; A) JB1101, which lacked the 31.8 and 29.1 MDa parental plasmids, B) L. lactis subsp. lactis 11454, C) JB1100, which lacked the 29.1 MDa parental plasmid, D-F) 11454, and G) E. coli V517 size standard plasmids (24). Size estimates of 11454 plasmid DNA from LeBlanc et al. (21).

dialyzed overnight against excess STC buffer (50 mM NaCl, 10mM Tris-Cl, pH 7.5, and 10mM $CaCl_2$).

Transductions were then performed between the phage preparations and L. lactis subsp. lactis strain LM0230.Selection upon BCP-glucose agar which contained 5 ug of nisin per ml or BCP-sucrose agar failed to identify any Nip^+, nisin-immune, or Suc^+ transductants.

Conjugation. Cell matings were performed between Nip^+Suc^+ donor strains and several Nip^-Suc^- recipients to determine whether transconjugants acquired novel plasmid DNA. Conjugation was detected between 11454 and Nip^-Suc^- L. lactis subsp. lactis recipients LM2301, LM2302, LM2306, JBR13, JB0213, KG491, and JK2301β. Plasmid analysis of at least 20 Nip^+Suc^+ transconjugants isolated from each of the recipients failed to identify any novel plasmid DNA in recipient backgrounds (e.g., Appendix Fig. 1). Similar results were obtained from transconjugants of the Nip^+Suc^+ donor L. lactis subsp. lactis 496 (data not shown). Conjugal exchange with strain DL16 was detected only with recipient JK2301β and Suc^+ transconjugants from this mating had all remained nisin sensitive (Nip^-). Plasmid analysis of these transconjugants also indicated that Suc^+ transfer was not accompanied by plasmid DNA (Appendix Fig. 1D). Transfer ability in L. lactis subsp. lactis 7962 was not detected with any of the recipients used in the study.

DNA-DNA hybridizations. Because plasmid curing data could not preclude the existence of an episome which encoded nisA, plasmid DNA was isolated from Nip$^+$Suc$^+$ donor strains 11454, 7962, DL16, and 496, and probed with the nisA-specific oligonucleotide. Autoradiography indicated that the probe could not hybridize to detectable plasmid DNA in the Nip$^+$Suc$^+$ strains (data not shown). To investigate a suspected chromosomal locus, genomic DNA was isolated from DL16 and the plasmid-cured derivative of 7962, JB704. The DNA was restricted with EcoRI and then DNA-DNA hybridizations were performed with the 5'-end labelled nisA oligonucleotide probe. As shown in Fig. 3, autoradiography indicated that nisA was located on a 10.5 kb EcoRI chromosomal fragment in JB704 and on 10.5 and 9.6 kb fragments in DL16.

Plasmid and chromosomal DNA preparations from Nip$^+$Suc$^+$ strains were also probed with plasmid pMH613 to determine whether the location of sucrose genes could be identified. Under conditions of moderate stringency, homology was not detected between pMH613 and plasmid DNA isolated from Nip$^+$Suc$^+$ strains 11454, 7962, or DL16 (Appendix Fig. 2A).

Results of hybridizations to chromosomal DNA isolated from the Suc$^-$ strain LM2306 and the Suc$^+$ transconjugant NS5406 were ambiguous. Homology between pMH613 and the lactococcal chromosomes was detected even at high stringency, but hybridization patterns on the autoradiogram were identical between LM2306 and NS5406 DNA preparations

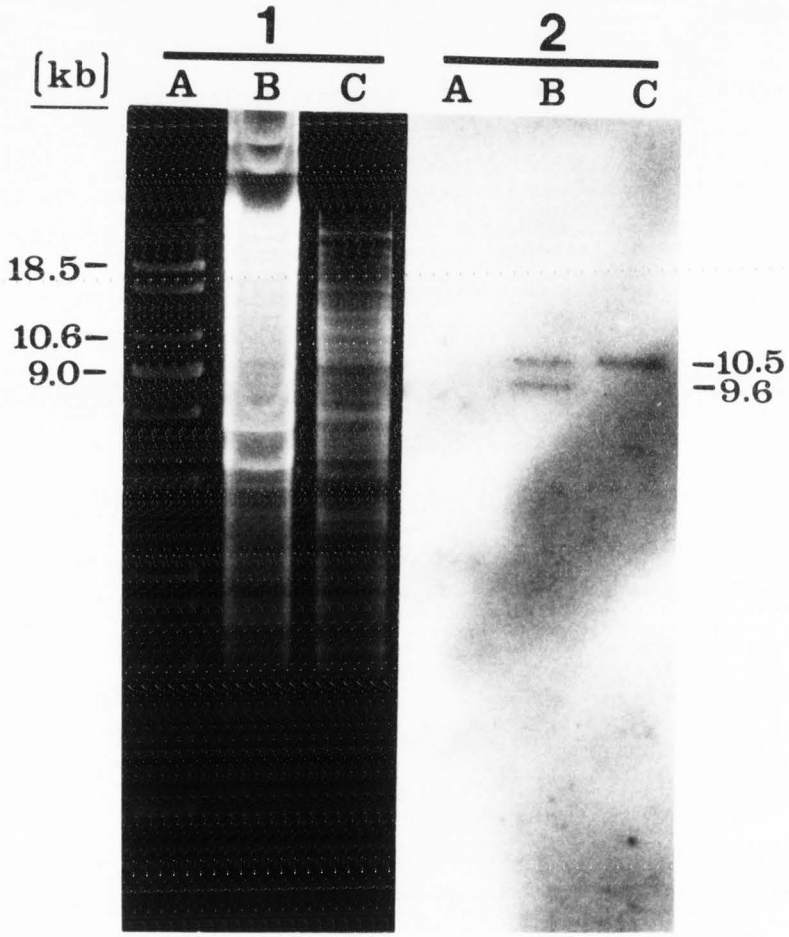

FIG. 3. DNA-DNA hybridization of EcoRI restricted genomic DNA isolated from L. lactis subsp. lactis DL16 and JB704 by an oligo nisA-specific probe. Panel 2 shows the autoradiogram obtained after a 72-h exposure of the blot, made from the agarose gel shown in panel 1, which was probed with the 5'-end labelled oligonucleotide. Hybridization was performed at room temperature for 24 h as described previously (4). DNA samples which corresponded to the lanes in each part were; A) Raoul™ I size standard preparation (American Synthesis Inc.), B) DL16 genomic DNA, and C) JB704 chromosomal DNA.

(Appendix Fig. 2A and 2B). In an attempt to clarify the ambiguity, the published restriction endonuclease map of pMH613 (35) was utilized to identify digests which would yield sucrose gene specific probes. Plasmid pMH613 was then restricted with PvuII to generate a 1.9 kb fragment which contained most of the Streptococcus mutans scrA gene, and double digested with BamHI and HindIII to provide a 0.6 kb fragment of the scrB gene. These fragments were purified from 0.8% agarose, 5'-end-labelled with [γ-^{32}P]ATP, and then utilized to probe restriction endonuclease digests of LM2306 and NS5406 chromosomal DNA. Results showed that under conditions of high stringency, the scrA probe yielded a homology pattern similar to that obtained from pMH613 (Appendix Fig. 2C and 2D) while hybridization to the scrB probe was not detected even at low stringency.

DISCUSSION

This study utilized plasmid curing, transduction, conjugation, and DNA-DNA hybridization to investigate purported plasmid loci for genes which encode Nip$^+$Suc$^+$ (12,20,37,45). Because of the relative ease with which plasmid DNA may be isolated and manipulated, identification of a Nip$^+$Suc$^+$ plasmid would facilitate molecular studies of nisin biosynthesis and the construction of novel nisin-producing strains for the dairy starter culture industry.

Evidence has been presented which indicated that the latter objective may be an effective method to reduce spoilage in cultured dairy products (15,23). Although several Nip^+Suc^+ strains were included in the study, substantial evidence had previously suggested Nip^+Suc^+ was encoded by plasmid DNA in strains 7962 and 11454 (12,20,37,45). Consequently, these strains were subjected to particular scrutiny.

Early genetics studies of lactococci successfully employed Arber (2) transduction experiments to distinguish between plasmid and chromosomally located genes for lactose and maltose utilization (27). For this reason, transductions were performed to determine whether Nip^+, nisin immunity or Suc^+ could be transduced from Nip^+Suc^+ parental strains 11454, 7962, and DL16, to LM0230. Although $CsCl_2$ density gradient tubes of phage preparation contained bands which may have indicated the presence of phage (17), transduction of Nip^+, Suc^+, or nisin immunity, was not detected. The presence of functional phage in the preparations could not be confirmed because indicator strains were not available. Consequently, the absence of detectable transduction may have been due to a number of factors including a predominance of defective particles in the preparations, or the absence of a generalized transducing phage.

Results from the plasmid curing studies, presented in Figs. 1 and 2 and Table 2, contradicted previous reports

which linked Nip^+Suc^+ to a 17.5 MDa plasmid in strain 7962 (45) and a 29.1 kb plasmid in strain 11454 (12,20,37). Orberg (31) first reported that L. lactis subsp. lactis 7962 contained 3 distinct plasmids sized at 13, 30, and 45 MDa. Tsai and Sandine (45), later reported that $CsCl_2$-purified preparations of 7962 plasmid DNA revealed additional molecules of 14.5, 17.5 and 18.5 MDa. They also presented data which suggested the 17.5 MDa plasmid encoded Nip^+Suc^+ and was conjugally transferred. When $CsCl_2$ preparations of 7962 plasmid DNA were analyzed in this study (Fig. 1), however, results agreed with the original data of Orberg (31). Furthermore, results from cell matings indicated that Nip^+Suc^+ in 7962 was not transferable.

Some of the discrepancies between these results and those of Tsai and Sandine (45) may be explained by re-examination of the data. The authors reported that a 14.5 MDa plasmid was present in 7962, yet this molecule was absent from the agarose gel presented in the study which described plasmid DNA of strain 7962. The report also indicated that the 17.5 and 18.5 MDa plasmids were obscured by chromosomal fragments in 0.7% agarose gels, thus $CsCl_2$ preparations of 7962 plasmid DNA were required to detect these molecules. Because covalently closed circular DNA migrates through agarose at a different rate than does linear DNA (25), manipulation of the agarose concentration

or electrophoresis time and voltage should allow resolution of plasmids from linear chromosomal fragments. Agarose gel electrophoresis of $CsCl_2$-purified 7962 plasmid DNA was performed in 0.6 and 0.8% gels for various times and voltages. Although the relative positions of the resident plasmids shifted with respect to the chromosomal fraction among these gels, no additional plasmid molecules were detected (data not shown). These results confirmed the original description of Orberg (31) which indicated that L. lactis subsp. lactis 7962 only contained plasmids of approximately 13, 30 and 45 MDa.

Plasmid curing results for 7962 also indicated that Nip^+Suc^+ was not associated with plasmid DNA in this strain. Nip^+Suc^- derivatives were isolated which had either no plasmid DNA or only one of the three parental plasmids (Fig. 1, Table 2). Similar results were obtained from plasmid-cured derivatives of L. lactis subsp. lactis 11454 (Fig. 2, Table 2). Curing studies by other investigators had linked the 29.1 MDa plasmid of this strain to Nip^+Suc^+ (12,20,37), but plasmid analysis of transconjugants had not confirmed this association. As shown in Fig. 2, derivatives of 11454 which lacked the 29.1 MDa plasmid were isolated in this study but these derivatives retained the Nip^+Suc^+ phenotype (Table 2). One explanation for this contradiction lies within the procedures utilized to investigate plasmid-cured

derivatives of 11454. Previous curing studies employed selection for Suc$^-$ derivatives prior to plasmid analysis (12,20,37). In contrast, this study utilized agarose gel electrophoresis to identify plasmid-cured derivatives before such strains were assayed for Nip$^+$Suc$^+$. Observations in this laboratory have suggested the 29.1 MDa plasmid was relatively unstable, thus previous reports which noted that Suc$^-$ isolates lacked this plasmid may have arisen from coincidental loss.

Although investigators previously reported novel plasmid DNA in lactococcal Nip$^+$Suc$^+$ transconjugants (11,45), plasmid analysis of several hundred transconjugants isolated in this study failed to indicate that Nip$^+$Suc$^+$ exchange was accompanied by plasmid DNA (e.g., Appendix Fig. 1). Similar results were obtained for Suc$^+$ exchange between DL16 and JK2301β. Conjugation studies indicated that strains 11454 and 496 were able to donate both Nip$^+$ and Suc$^+$, while DL16 transferred Suc$^+$ only and 7962 lacked any detectable conjugative ability. Examination of these cultures in M17-L broth demonstrated that 11454 and 496 exhibited a cell aggregation phenotype similar to that linked previously with high-frequency lactose plasmid transfer in some lactococci (47,48). Although Nip$^+$Suc$^+$ transfer from 11454 and 496 donors did not occur at high-frequency, cell aggregation in these strains may be associated with the ability to transfer

Nip$^+$Suc$^+$.

Because plasmid curing and conjugation studies could not preclude the possibility that Nip$^+$Suc$^+$ was encoded by an episome which had integrated into the chromosome, DNA-DNA hybridizations to total plasmid DNA isolated from Nip$^+$Suc$^+$ strains 7962 and 11454 were performed with the nisA oligonucleotide probe. Autoradiography indicated that the probe could not hybridize to plasmid DNA in either strain, which provided further evidence against plasmid involvement (data not shown). DNA-DNA hybridizations between the nisA probe and plasmid DNA isolated from strains 496 and DL16 also failed to demonstrate a plasmid locus for this gene. Alternatively, strong evidence for a chromosomal nisA locus was obtained from DNA-DNA hybridizations to EcoRI restricted genomic and chromosomal DNA preparations isolated from Nip$^+$Suc$^+$ strains. Data presented in Fig. 3 demonstrated that the nisA gene was located within EcoRI chromosomal fragments of approximately 10.5 kb in DL16 and JB704. Previous results have demonstrated that nisA was located upon chromosomal EcoRI fragments of similar size in the 11454 transconjugant NS5406 (4) and a L. lactis subsp. cremoris transconjugant which acquired Nip$^+$Suc$^+$ from strain 496 (unpublished data). Figure 3 also shows that the probe hybridized to an additional fragment of 9.6 kb in DL16. Since the 14-mer oligonucleotide does not contain an EcoRI recognition

sequence within it, the 9.6 kb band may represent a second copy of nisA in this strain.

The conclusion that Nip$^+$Suc$^+$ was encoded by chromosomal loci agreed with recent reports by Rauch et al. (33), Steen et al. (39), and Horn et al. (14). These studies utilized pulsed-field gel electrophoresis (PFGE) to analyze genomic DNA isolated from 11454 and Nip$^+$Suc$^+$ transconjugants of L. lactis subsp. lactis MG1614 constructed from different Nip$^+$Suc$^+$ donors. Horn et al. (14) also showed that within the chromosome of several Nip$^+$Suc$^+$ strains, these genes are encoded by Tn5301, a conjugative transposon approximately 70 kb in length. Furthermore, this laboratory noted that a comparison between PFGE data of Steen et al. (39) and Tulloch et al. (46), allowed nisA to be mapped upon the 11454 chromosome.

Although Tn5301 may be widespread among Nip$^+$Suc$^+$ lactococci (14), conjugation results from this study suggested that this element either was not present or did not function in strains 7962 and DL16. The studies indicated that 7962 lacked conjugative ability and that in DL16 this capability was limited to Suc$^+$ transfer which was detected only with recipient JK2301β. Suc$^+$Nip$^-$ transconjugants were rarely isolated from 11454 (Broadbent and Kondo, unpublished data), thus the observation that all transconjugants of DL16 exhibited this phenotype indicated that the conjugative element in this donor differed from

those of 11454 and 496. It was interesting to note that Suc^+ transfer from DL16 was only detected among recipients which contained the self-transmissible plasmid pAMβ1 (22). A previous report of Suc^+ transfer from DL16 also was limited to a recipient which contained a conjugative plasmid (37). These data provided evidence to suggest that genes for conjugation in these recipients functioned *in trans* to permit Suc^+ transfer from donor strain DL16.

Lactococci utilize sucrose via a PEP-PTS system (42,43), and amino acid sequence homology has been reported among PEP-PTS sugar-specific permeases of various gram-positive and gram-negative bacteria (34,35). Attempts to locate lactococcal sucrose genes through DNA-DNA hybridization to *scrA* and *scrB* genes for PEP-PTS utilization of sucrose in *Streptococcus mutans* (35), however, were not successful. Results indicated that considerable homology existed between the *S. mutans scrA* gene in pMH613 and NS5406 and LM2306 lactococcal genomic DNA, but the homology could not be attributed to the presence of a lactococcal *scrA* gene. Instead, extensive yet identical hybridization was detected in chromosomal DNA preparations of the Suc^+ transconjugant NS5406 and the Suc^- recipient LM2306 (Appendix Fig. 2). Since lactococci utilize a number of carbohydrates by PEP-PTS (42), and amino acid sequence homology has been noted between enzyme-II permeases for different carbohydrates (34,35), the

results may indicate DNA homology between the S. mutans E-II^{scr} gene and those for a number of lactococcal sugar-specific permeases.

The ambiguous results obtained from hybridization of the S. mutans PEP-PTS sucrose genes to lactococcal DNA supported a recent report by Thompson et al. (44). To overcome a similar difficulty, these authors sequenced the amino terminal ends of lactococcal sucrose-6-phosphate hydrolase and E-II^{scr} enzymes, then constructed degenerate oligonucleotide gene probes based upon those sequences. Results from that study indicated that genes for sucrose utilization resided in the chromosome of L. lactis subsp. lactis strains K1-23 and 11454 and that these genes were not present in Suc$^-$ lactococci (44).

In conclusion, plasmid curing, conjugation and DNA-DNA hybridization data obtained in this study contradicted earlier reports which linked Nip$^+$Suc$^+$ to plasmid DNA in L. lactis subsp. lactis strains 7962 and 11454 (12,20,37,45). Instead, the cumulative evidence suggested chromosomal loci encoded these genes in all strains examined. Conjugation results for strains 11454 and 496 detected Nip$^+$Suc$^+$ transfer to the Rec$^-$ recipient JBR13, but novel plasmid DNA could not be detected in this or any other recipient utilized in the study (Appendix Fig. 1). These observations were consistent with previous data (12,37) and supported reports which have indicated that Nip$^+$Suc$^+$ was

encoded by a conjugative transposon (14,33,44). The absence of conjugative ability in 7962 and atypical Suc^+ transfer only from DL16, suggested that such an element may either be lacking or nonfunctional in these strains. Although we concluded that Nip^+Suc^+ was chromosomally encoded in strains 7962, 11454, 496, and DL16, these genes may be encoded by plasmid DNA in other nisin-producing strains such as 6F3 (16). The potential advantages offered by the isolation and manipulation of a Nip^+Suc^+ plasmid will likely promote further inspections of Nip^+Suc^+ lactococci.

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CHAPTER III

GENETIC CONSTRUCTION OF NISIN-PRODUCING LACTOCOCCUS LACTIS SUBSP. CREMORIS AND ANALYSIS OF A RAPID METHOD FOR CONJUGATION[1]

ABSTRACT

Conjugation was used to construct nisin-producing Lactococcus lactis subsp. cremoris strains. Recipients were obtained by electroporation of L. lactis subsp. cremoris strains with the drug resistance plasmid pGK13 or pGB301. A method, direct-plate conjugation, was developed in which donor and recipient cells were concentrated and then combined directly on selective media. This method facilitated transfer of the nisin-sucrose (Nip^+Suc^+) phenotype from the donor strain, L. lactis subsp. lactis 11454, to three L. lactis subsp. cremoris recipient strains. Nip^+Suc^+ L. lactis subsp. cremoris transconjugants were obtained at frequencies which ranged from 10^{-7} to 10^{-8} per donor CFU. DNA-DNA hybridization to transconjugant DNAs, performed with an oligonucleotide probe synthesized to detect the nisin precursor gene,

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showed that this gene was transferred during conjugation but was not associated with detectable plasmid DNA. Further investigation indicated that Lactococcus lactis subsp. cremoris Nip^+Suc^+ transconjugants retained the recipient strain phenotype with respect to bacteriophage resistance and acid production in milk. Results suggested that it would be feasible to construct nisin-producing L. lactis subsp. cremoris strains for application as mixed and multiple starter systems. Additionally, the direct-plate conjugation method required less time than filter or milk agar matings and may also be useful for investigations of conjugal mechanisms in these organisms.

INTRODUCTION

Nisin is a bacteriocin produced by some strains of Lactococcus lactis subsp. lactis. It inhibits a variety of gram-positive bacteria (21) and has been used frequently, in Europe, as an additive to prevent clostridial blowing in processed cheese (24). The Food and Drug Administration recently awarded a nisin preparation generally regarded as safe (GRAS) status within the United States for use in certain pasteurized cheese spreads to prevent contamination by Clostridium botulinum (13).

Investigators have attempted to use nisin-producing starter cultures, alone or with the usual industrial starter strains of lactococci, lactobacilli, and

propionibacteria, to control clostridial spoilage in rennet-set Dutch and Swiss cheeses (Edam, Emmental). The nisin producers controlled clostridial growth but adversely affected cheese quality even when the industrial starters were included, because they were also inhibited by nisin (21,24). Later experiments which combined nisin producers with nisin-resistant mutants of the industrial starters also proved ineffective because the latter organisms suffered from prolonged lag phase, increased bacteriophage sensitivity, lower heat resistance and lower acid production in milk (24). The nisin sensitivity of cultures used in mixed and multiple starter systems has continued to prevent the inclusion of a single nisin-producing strain and has limited the use of nisin to direct addition in processed products. Nisin-producing cheese starters have remained appealing, however, because the bacteriocin has been shown to inhibit pathogenic and spoilage organisms such as Listeria, Staphylococcus, and Clostridium spp., which occasionally contaminate cheeses (5,7,21,24). Additionally, U.S. cheese processors that utilized cheeses made with nisin-producing starters could reduce the amount of added nisin needed in cheese spread blends. Genetics studies have recently suggested that it may be possible to construct nisin-producing starter cultures which would avoid the problems observed by early investigators.

Although the nisin precursor gene, nisA, has been cloned (6,9,22), nisin production by clones has not been reported. Expression will likely require cloning of the gene(s) needed for nisin activation (21,9) and nisin immunity (if the cloning host is sensitive). Gasson (15) first reported conjugal transfer of nisin production and immunity (Nip^+) among strains of L. lactis subsp. lactis and the simultaneous transfer of sucrose-fermenting ability (Suc^+). Gonzales and Kunka (17) later described transfer of the Nip^+Suc^+ phenotype into a strain of L. lactis subsp. lactis biovar. diacetylactis and discovered that the transconjugant had acquired resistance to a lytic bacteriophage. Murphy et al. (29) also reported reduced bacteriophage sensitivity (Rbs^+) in Suc^+ transconjugants of L. lactis subsp. lactis. Steele and McKay, in 1986 (35), reported on the genetic basis of Nip^+Suc^+ and mentioned conjugal transfer of these genes into a strain of L. lactis subsp. cremoris. These reports indicated that natural gene transfer might be utilized to obtain Nip^+ starters.

This report describes the method of direct-plate conjugation (DPC), a technique which enhanced transfer of Nip^+Suc^+ among L. lactis subsp. lactis strains. When DPC was applied to three L. lactis subsp. cremoris recipients obtained by transformation of parental strains with drug resistance plasmids, Nip^+Suc^+ transconjugants from all three recipients were obtained. These transconjugants

retained the recipient strain phenotype with respect to bacteriophage resistance and acid production in milk. DNA-DNA hybridization data also indicated that the nisA gene was transferred during conjugation but was not associated with detectable plasmid DNA in either donor or transconjugant cells. Finally, the DPC method was used to investigate features of Nip^+Suc^+ transfer.

MATERIALS AND METHODS

Bacterial strains. Lactococcal strains used in this study are described in Table 1. Cultures were stored at 4°C and maintained by biweekly transfers in M17 broth (38), which contained 0.5% glucose or lactose (M17-G or M17-L), as the sole carbohydrate source. Lactococcal cultures were grown at 30°C. Escherichia coli strain V517 (25) was grown in Brain-Heart Infusion (BBL Microbiology Systems, Cockeysville, Md.) at 37°C with aeration.

Electroporation. To develop suitable L. lactis subsp. cremoris recipients for conjugation, strains were transformed by electroporation with either the 4.9 kb plasmid pGK13 (37) or the 9.8 kb plasmid pGB301 (4). These plasmids each contain resistance determinants to erythromycin (Em) and chloramphenicol which are expressed in lactococcal hosts. Cells for electroporation were grown to an optical density at 600 nm of 0.3 in M17-L, centrifuged at 4300 x g for 10 min, washed with 5 ml of ice cold electroporation buffer (EB; 1 mM potassium phosphate

TABLE 1. Lactococcal strains used in the study

Strain	Relevant phenotype[a]	Description (reference)
L. lactis subsp. *lactis*:		
ATCC 11454	$Nip^+Suc^+Em^s$	Nip^+Suc^+ donor (35).
ML3	Lac^+Em^s	Lac^+ donor (2).
LM2306	$Nip^-Suc^-Lac^-Em^r$	Plasmid-cured recipient derived from strain C2 (35).
NS5406	$Nip^+Suc^+Em^rRbs^+$	Nip^+Suc transconjugant from 11454 x LM2306 (this study).
JBML06	Lac^+Em^r	Lac^+ transconjugant from ML3 x LM2306 (this study).
7962	$Nip^+Suc^+Em^s$	Nip^+Suc^+ donor (40).
DL16	$Nip^+Suc^+Em^s$	Nip^+Suc^+ donor (35).
L. lactis subsp. *cremoris*:		
CS224	$Lac^+Nip^-Suc^-Em^s$	Parental strain (43).
SW224	$Lac^+Nip^-Suc^-Em^r$	CS224 transformed with pGB301 by protoplast transformation (43).
NS224	$Lac^+Nip^+Suc^+Em^r$	Nip^+Suc^+ transconjugant from 11454 x SW224 (this study).
EB7	$Lac^+Nip^-Suc^-Em^s$	Parental strain (33).
JKEB7	$Lac^+Nip^-Suc^-Em^r$	EB7 electrotransformed with pGB301 (this study).
NSEB7	$Lac^+Nip^+Suc^+Em^r$	Nip^+Suc^+ transconjugant from 11454 x JKEB7 (this study).
C3	$Lac^+Nip^-Suc^-Em^s$	Parental strain (33).
JBC3	$Lac^+Nip^-Suc^-Em^r$	C3 electrotransformed with pGK13 (this study).
NSC3	$Lac^+Nip^+Suc^+Em^r$	Nip^+Suc^+ transconjugant from 11454 x JBC3 (this study).

[a]Abbreviations: Nip^+, nisin production and immunity; Suc^+, sucrose utilization; Lac^+, lactose utilization; Em^r, erythromycin resistant; Rbs^+, reduced bacteriophage sensitivity

buffer, pH 7.4, 0.5 M sucrose, 1 mM $MgCl_2$) (31), and suspended in 0.01 culture volume of cold EB. Cells (200 ul) were then mixed with 1 ug of $CsCl_2$-purified plasmid DNA (in 10mM Tris-hydrochloride, pH 8.0) and loaded into the 2.5 mm electroporation cuvette of a Prototype Design Services Model ZA1000 electroporation unit (Madison, Wis.). A single pulse of 1.75 kV (7.0 kV/cm, 15 uF capacitance) was delivered to the mixture, then cells were cooled on ice for 10 min, 200 ul of cold 2X M17-G was added, and the mixture chilled on ice an additional 15 min. The cells were incubated for 1 h to allow expression of the erythromycin resistance (Em^r) determinant and then plated on M17-G agar which contained 0.5 M sucrose and 5 ug of erythromycin per ml. Plates were examined for Em^r colonies after 48 h of incubation and agarose gel electrophoresis was used to detect pGK13 or pGB301 in lysates prepared from the putative transformants.

Direct-plate conjugal matings. Observations in this laboratory indicated that conjugation would occur if donor and recipient cells were mixed directly on selective media. To prepare cells for conjugation, 1.5% inoculations were made into fresh broth from 18 h cultures. Donors were grown in M17-L broth and recipients in M17-G or M17-L which contained 5 ug of erythromycin per ml. Donor and recipient cultures were incubated for 4 h (>10^7 CFU/ml), collected by centrifugation at 4300 x g, washed in 5 ml of 0.85% saline

(Nip^+Suc^+ donor cells were washed three times to remove residual nisin), suspended in 0.1 volume of saline, and placed on ice. Donors and recipients were then mixed 1:2, to a final volume of 100 ul, directly on selective media. Conjugal transfer of lactose-fermenting ability (Lac^+) was performed on bromocresol purple (BCP)-lactose indicator agar (28) which contained 5 ug/ml Em, and transfer of Nip^+Suc^+ was performed on BCP-sucrose agar (35) that contained the antibiotic. Donor and recipient controls received the respective cell ratio blended with saline. After 48 h of incubation, plates were examined for large, yellow, transconjugant colonies (e.g., Fig. 1) and transconjugants were verified by phenotypic characterization and plasmid analysis. Transfer frequencies were expressed as the number of transconjugants per donor CFU and the values reported were the average of at least four separate experiments.

Controls against other forms of gene transfer were performed to verify that conjugation was the mechanism of transfer on selective media. Transduction controls were performed by blending filtered (0.45 um) donor growth supernatant with recipient cells (1:1), adding 1 M $CaCl_2$ to a final concentration of 10 mM, and plating the mixture (100 ul) onto selective media. For transformation controls, 0.5 ml each of donor and recipient cell preparation was placed into sterile 1.5 ml microcentrifuge

tubes and centrifuged for 10 s at 16,000 x g to pellet cells. The donor and recipient cell pellets were each suspended in 0.5 ml of filter-sterilized 0.85% saline which contained 100 mg of DNase I (Sigma Chemical Co., St. Louis, Mo.) per ml, and matings were performed as described above. Conjugation controls were performed with non-viable donor cells prepared by two methods; heating for 1 h at 55°C, and the Clorox treatment method described by Hershfield (18). Treated cells were plated onto Elliker's (Difco Laboratories, Detroit, Mich.) agar to confirm donor cell death.

The cross-streak DPC assay was developed as a modification of the Franke and Clewell (14) procedure for detection of high-frequency conjugal events. Donor and recipient cells were streaked perpendicular to one another on selective media with sterile cotton applicators. Three potential high frequency donor strains with a common recipient were assayed on each plate. Plates were examined for transconjugant colonies after 48 h of incubation and transconjugants were verified by phenotypic characterization and plasmid analysis.

Solid surface milk agar conjugation. Solid surface milk agar matings were performed by the method of McKay et al. (27), except that for $Nip^{+}Suc^{+}$ matings, the α-chymotrypsin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) treatment of Steele and McKay (35) was

used. Transconjugants from solid surface milk agar matings were detected either by replica plating onto selective media (2) or cells were harvested from the milk agar plate (27) in 1 ml of 0.85% saline and then 0.1-ml volumes were plated onto selective media. Transfer frequencies were calculated as described above for DPC matings.

DNA isolation and purification. Plasmids were isolated by the method of Anderson and McKay (1) and, if needed, purified by $CsCl_2$ density gradient centrifugation (26). The presence of plasmids in the cell lysates was established by electrophoresis in 0.6% agarose gels at 3 V/cm for 7 h with $CsCl_2$-purified plasmids from E. coli V517 (25) included as plasmid size standards.

Genomic DNA was isolated by a modification of the Anderson and McKay procedure (1). After sodium dodecyl sulfate was added, the cell mixture was vortexed at high speed for 15 s. NaCl was added to a final concentration of 3%, and then the solution was extracted with 1 volume of chloroform-isoamyl alcohol (24:1). The aqueous phase was collected after centrifugation at 4000 x g for 10 min at 4°C, and the extraction was repeated. DNA was precipitated with 2 volumes of absolute ethanol and stored overnight at -20°C. The DNA was collected by centrifugation at 4°C for 20 min at 7600 x g, dried, and dissolved in 10 mM Tris-HCl, pH 8.0. Residual protein was removed by addition of an equal volume of 5 M ammonium acetate followed by

centrifugation at 16,000 x g for 15 min. The supernatant was collected and the DNA was precipitated with ethanol. After centrifugation the pellet was washed with 70% ethanol, dried, and suspended in 5 ml of TE buffer (10 mM Tris, pH 8.0, 1mM EDTA).

Restriction analysis of genomic DNA was performed according to the enzyme manufacturer's directions (Bethesda Research Laboratories, Inc., Gaithersburg, Md., or International Biotechnologies Inc., New Haven, Conn.). Prior to electrophoresis, 3 ul of RNase A (Sigma Chemical Co.) (1 mg/ml in 10 mM Tris-HCl, pH 8.0) was added to each sample. Restricted genomic DNA was separated in 0.8% agarose gels at 1.4 V/cm for 18 h. Adenovirus type 2 DNA, digested with EcoRI and BamHI (International Biotechnologies Inc.), was used for fragment size standards.

Oligonucleotide probe synthesis and DNA-DNA hybridizations. To detect the nisA gene in cells, a 14-mer oligonucleotide probe was synthesized (Utah State University Biotechnology Center, Logan) with an Applied Biosystems Model 380B DNA synthesizer (Foster City, Calif.) and then purified by high-pressure liquid chromatography. The synthetic oligonucleotide, 5'-ATGTTACAACCCAT-3', was complementary to a portion of the published nisA gene sequence of L. lactis subsp. lactis 11454 (6,9,22). 5'-end labelling of the nisA probe with [γ-^{32}P]ATP, hybridization

to plasmid and genomic DNA in NaCl-sodium citrate solution, and autoradiography were performed as described by Ausubel et al. (3). Transfer of DNA to GeneScreen*Plus*™ nylon membranes (E.I. du Pont de Nemours & Co., Inc., NEN Research Products, Boston, Mass.) was performed by the method of Southern (34).

Assays. Nisin production by transconjugants was verified by the agar overlay method described by Steele and McKay (35). To determine whether Nip^+Suc^+ transconjugants expressed Rbs^+, spot assays for bacteriophage sensitivity were performed as described by Terzaghi and Sandine (38). Fast acid production in milk was measured by the ability to clot 11% nonfat dry milk (NDM), at 21°C, within 16 to 18 h.

RESULTS

Development of DPC and methods comparison. Transfer frequencies of the Lac^+ and Nip^+Suc^+ phenotypes that were obtained from DPC and solid surface milk agar matings (27) were compared. The recipient for both matings was *L. lactis* subsp. *lactis* LM2306 (35). The Lac^+ donor was *L. lactis* subsp. *lactis* ML3 (2), and *L. lactis* subsp. *lactis* 11454 (17,35) served as the Nip^+Suc^+ donor. Results of the comparison are shown in Table 2. Direct-plate conjugation provided a similar rate of Lac^+ transfer but produced a 4- to 100-fold-higher frequency for Nip^+Suc^+ transfer. Plasmid analysis of transconjugants showed that all Lac^+ isolates screened contained either the 33 MDa Lac plasmid

TABLE 2. Comparison of conjugal transfer frequencies obtained by DPC and milk agar plate methods

Mating donor x recipient	Selected phenotype[a]	Method	Transfer frequency transconjugants/donor CFU
ML3 x LM2306	Lac^+Em^r	Milk agar; RP[b]	2.2×10^{-9}
ML3 x LM2306	Lac^+Em^r	Milk agar; harvest[c]	1.1×10^{-7}
ML3 x LM2306	Lac^+Em^r	DPC	1.1×10^{-7}
ML3 filtrate x LM2306	Lac^+Em^r	DPC	$<7.2 \times 10^{-10}$
ML3+DNAse x LM2306+DNAse	Lac^+Em^r	DPC	6.2×10^{-8}
Non-viable ML3[d] x LM2306	Lac^+Em^r	DPC	$<7.2 \times 10^{-10}$
ATCC 11454 x LM2306	Suc^+Em^r	Milk agar; RP[b]	2.4×10^{-8}
ATCC 11454 x LM2306	Suc^+Em^r	Milk agar; harvest[c]	1.6×10^{-6}
ATCC 11454 x LM2306	Suc^+Em^r	DPC	7.0×10^{-6}
ATCC 11454 filtrate x LM2306	Suc^+Em^r	DPC	$<1.9 \times 10^{-9}$
ATCC 11454+DNAse x LM2306+DNAse	Suc^+Em^r	DPC	8.7×10^{-7}
Non-viable ATCC 11454[d] x LM2306	Suc^+Em^r	DPC	$<1.9 \times 10^{-9}$

[a]Abbreviations: Suc^+, sucrose utilization; Lac^+, lactose utilization; Em^r, erythromycin resistance.
[b]Transconjugants detected by replica-plating onto selective media (2).
[c]Cell mixture was harvested from the milk agar plate with 1-ml 0.85% saline and then cells were plated on selective media to detect transconjugants (27).
[d]Donors heat-killed (55°C for 1 h) or chlorox treated (18).

and/or the 60 MDa cointegrate (2), while Suc$^+$ isolates contained no detectable plasmid DNA (35). When assayed, over 30 Suc$^+$ transconjugants were all found to be Nip$^+$ (e.g., Appendix Fig. 3).

Gene transfer controls indicated transfer had not occurred with non-viable donors or filtered growth supernatant, and was not eliminated by DNAse I (Table 2). The mechanism of gene transfer detected on antibiotic-containing media was therefore conjugation.

An example of the cross-streak DPC assay for high-frequency Nip$^+$Suc$^+$ transfer is shown in Fig. 1. The results demonstrated that the transfer of Nip$^+$Suc$^+$ from 11454 to LM2306, which occurred at a frequency of 7×10^{-6} transconjugants per donor CFU with DPC (Table 2), could be detected by the assay. Nip$^+$Suc$^+$ transfer from L. lactis subsp. lactis donors DL16 (35), or 7962 (40), to LM2306 was not demonstrated by any of the conjugal techniques used in this study.

Although transfer of Lac$^+$ from ML3 to LM2306 was not detected by cross-streak DPC, experiments in this laboratory have shown that the assay readily identified Lac$^+$ transconjugants that subsequently became high-frequency donors of this trait (16,41; data not shown).

Conjugation of Nip$^+$Suc$^+$ into L. lactis subsp. cremoris. To obtain recipients for conjugation, Lactococcus lactis subsp. cremoris strain EB7 (33) was transformed by electroporation with the plasmid pGB301, and

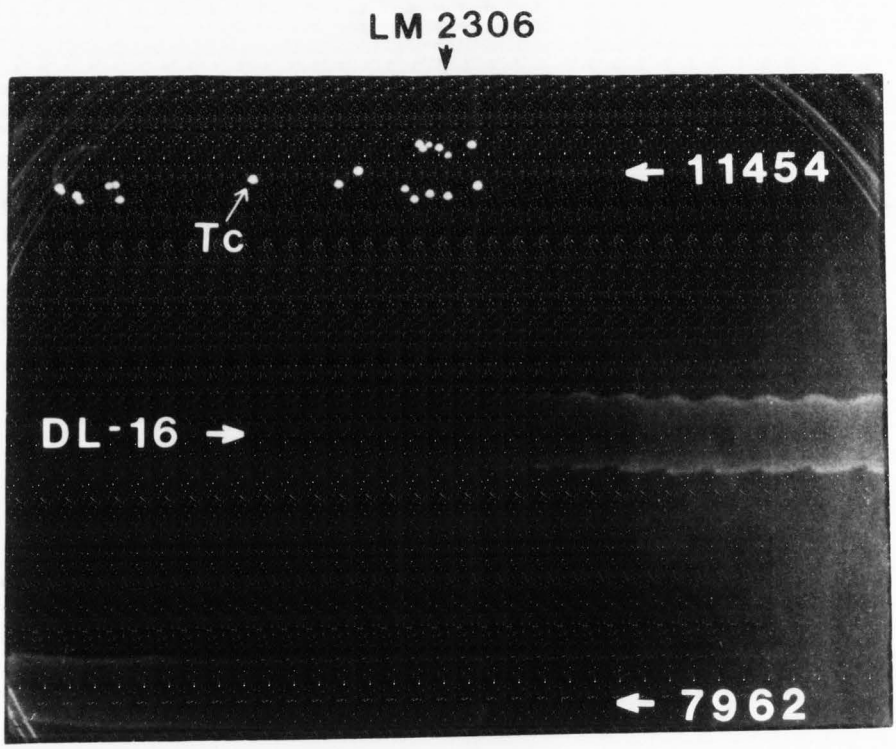

FIG. 1. Cross-streak DPC assay for high-frequency $Nip^{+}Suc^{+}$ transfer. Arrows next to strain designations indicate the direction cells were streaked with sterile cotton applicators. The recipient, LM2306, was streaked first and then donors were streaked perpendicular to the recipient. The bromocresol-sucrose plate, which contained 5 ug of erythromycin per ml, was photographed after 48 h incubation at 30°C. Tc, $Suc^{+}Em^{r}$ transconjugant colony.

strain C3 (33) was electrotransformed with plasmid pGK13. Protoplast transformation of strain CS224 with pGB301 was described previously (43). The L. lactis subsp. cremoris transformants, SW224, JKEB7, and JBC3, all expressed resistance to 5 ug of erythromycin per ml in broth, and plasmid profiles revealed a new plasmid band which comigrated with the respective $CsCl_2$-purified drug resistance plasmid during agarose gel electrophoresis (e.g., Fig. 4).

DPC was used for matings between the Nip^+Suc^+ donor L. lactis subsp. lactis 11454 and the L. lactis subsp. cremoris transformants SW224, JKEB7, and JBC3. Suc^+ transconjugants were obtained which were also Nip^+ (Fig. 2). The frequency of Nip^+Suc^+ transfer ranged from slightly under 4.0×10^{-7} transconjugants per donor CFU for L. lactis subsp. cremoris recipient strains SW224 and JBC3, to 4.5×10^{-8} per donor CFU for strain JKEB7. When solid surface milk agar matings were performed, only recipient SW224 yielded Nip^+Suc^+ transconjugants. The frequency of transfer, 1.4×10^{-8}, was 25X lower than that obtained by DPC.

Characteristics of Nip^+Suc^+ transconjugants. Reduced bacteriophage sensitivity in Nip^+Suc^+ transconjugants of L. lactis subsp. lactis has been reported previously (17,29). The bacteriophage spot assays detected a difference in phage sensitivity between the L. lactis subsp. lactis recipient LM2306, and the Nip^+Suc^+ transconjugant of that

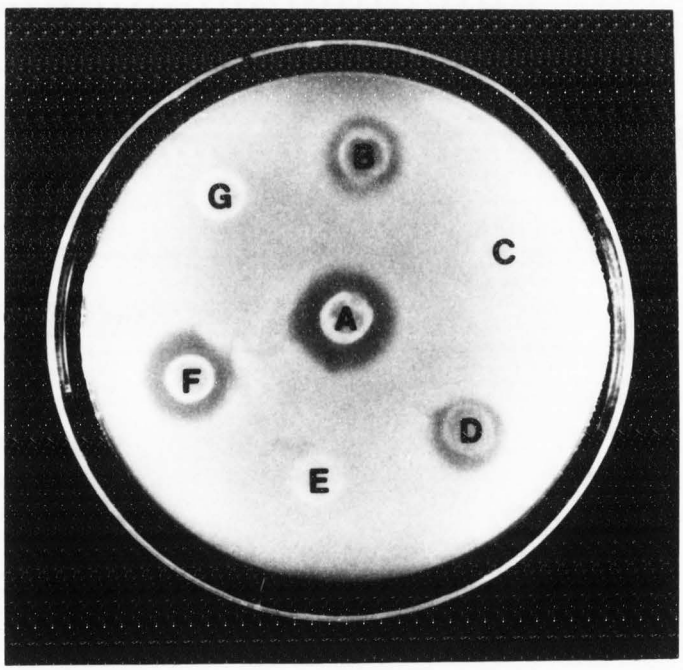

FIG. 2. Agar overlay assay for nisin production. Included in the assay were Nip$^+$Suc$^+$ donor L. lactis subsp. lactis 11454 (A), L. lactis subsp. cremoris Nip$^+$Suc$^+$ transconjugant NS224 (B), L. lactis subsp. cremoris parental CS224 (C), L. lactis subsp. cremoris Nip$^+$Suc$^+$ transconjugant NSC3 (D), L. lactis subsp. cremoris parental C3 (E), L. lactis subsp. cremoris Nip$^+$Suc$^+$ transconjugant NSEB7 (F), and L. lactis subsp. cremoris parental EB7 (G).

strain, NS5406. Phage c2 (4.5×10^7 PFU/ml) formed a zone of inhibition on a lawn of L. lactis subsp. lactis LM2306 cells, while no zone of inhibition was observed when the phage was spotted onto lawns of NS5406 or 11454. This result indicated that the L. lactis subsp. lactis transconjugant acquired Rbs^+ with Nip^+Suc^+. Alterations in bacteriophage sensitivity patterns were not detected among the L. lactis subsp. cremoris recipients and Nip^+Suc^+ transconjugants.

To further characterize L. lactis subsp. cremoris Nip^+Suc^+ transconjugants, acid production in milk was examined. All L. lactis subsp. cremoris parentals and Nip^+Suc^+ transconjugants were initially fast acid producers. Strains CS224 and C3, however, slowly lost this capability after repeated transfer in nonfat dry milk (28) and Nip^+Suc^+ transconjugants of these strains behaved similarly. L. lactis subsp. cremoris EB7 and Nip^+Suc^+ transconjugants of this strain remained fast.

Finally, DNA-DNA hybridizations were performed to determine whether the nisA gene was transferred during conjugation and if it could be detected on plasmid DNA. Restriction digests with the enzymes ClaI, EcoRI,and HindIII, were performed on genomic DNA isolated from L. lactis subsp. lactis LM2306 and the Nip^+Suc^+ transconjugant of that strain, NS5406. The DNA fragments were separated in an agarose gel and then transferred to a hybridization

membrane and probed with the 5'-end-labelled oligonucleotide constructed to detect the nisA gene. Autoradiography showed the probe had hybridized to DNA fragments in the Nip^+Suc^+ transconjugant, NS5406, but did not hybridize to Nip^-Suc^- LM2306 recipient DNA. The bands observed on the autoradiogram (Fig. 3) correlated with NS5406 DNA fragments of approximately 35.9, 8.5, and 4.2 kilobases (kb), respectively, in ClaI, EcoRI, and HindIII digests. To investigate whether the nisA gene might be plasmid associated, plasmid DNA was isolated from the L. lactis subsp. cremoris recipients, their Nip^+Suc^+ transconjugants, and the Nip^+Suc^+ donor L. lactis subsp. lactis 11454 (Fig. 4). Visual examination of the ethidium-bromide stained agarose gel could not correlate the exchange of Nip^+Suc^+ from 11454 with the appearance of a new plasmid molecule in the L. lactis subsp. cremoris recipients. Despite this result, the plasmid DNAs were transferred to a hybridization membrane and then probed with the oligonucleotide. Autoradiography demonstrated that the probe had hybridized to chromosomal DNA bands of the Nip^+Suc^+ donor and L. lactis subsp. cremoris transconjugants but hybridization to plasmid DNA was not detected (data not shown).

Investigation of Nip^+Suc^+ conjugation. Modifications to DPC and solid-surface milk agar matings were performed to investigate events which affected Nip^+Suc^+ transfer (Table 3). To determine whether transfer could occur if

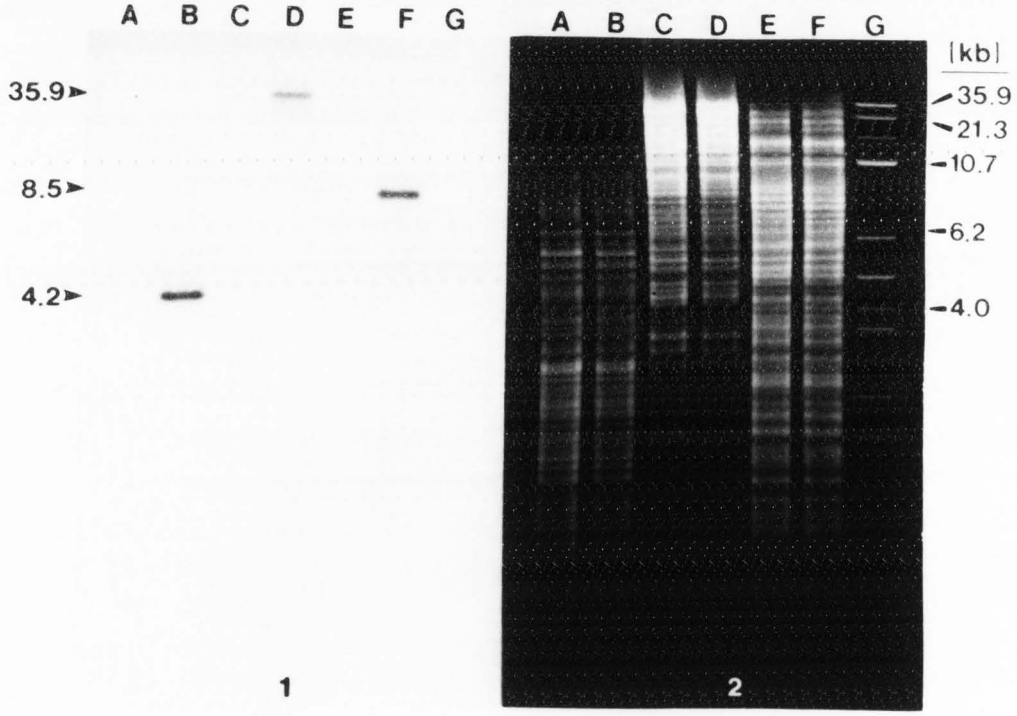

FIG. 3. Hybridization of restricted genomic DNA from L. lactis subsp. lactis recipient LM2306 and its' $Nip^{+}Suc^{+}$ transconjugant, NS5406, by an oligo nisA-specific probe. Panel 1 shows the autoradiogram obtained after a 72-h exposure of the blot, made from the agarose gel shown in panel 2, which was probed with the labelled oligonucleotide. Hybridization was performed at room temperature for 24 h as described by Ausubel et al. (3). DNA samples which corresponded to the lanes in each part were; A) HindIII digested LM2306, B) HindIII digested NS5406, C) ClaI digested LM2306, D) ClaI digested NS5406, E) EcoRI digested LM2306, F) EcoRI digested NS5406, and G) BamHI/EcoRI digested Ad2 DNA fragment size standards.

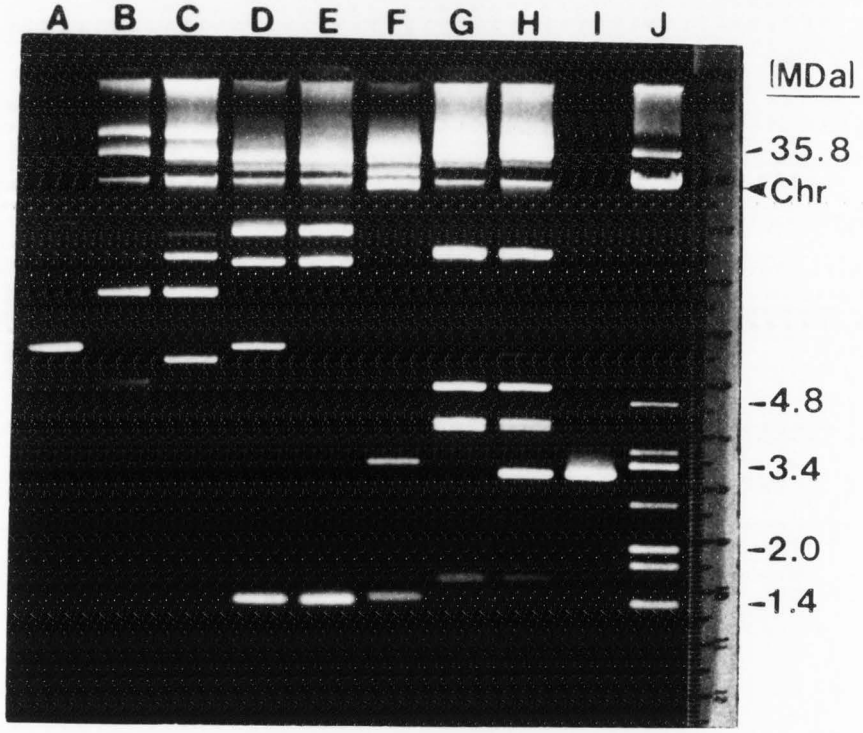

FIG. 4. Agarose gel electrophoresis of plasmids isolated from L. lactis subsp. cremoris parental strains, the Nip$^+$Suc$^+$ transformant-transconjugants, and the Nip$^+$Suc$^+$ donor. The transconjugants were obtained after mating the Nip$^+$Suc$^+$ donor with parental strains which had been transformed with either pGB301 or pGK13, as described in the text. Lanes: A, CsCl purified pGB301 (4); B, L. lactis subsp. cremoris Nip$^+$Suc$^+$ transconjugant NSEB7, which contained pGB301; C, L. lactis subsp. cremoris parental EB7; D, L. lactis subsp. cremoris Nip$^+$Suc$^+$ transconjugant NS224, with pGB301; E, L. lactis subsp. cremoris parental CS224; F, Nip$^+$Suc$^+$ donor L. lactis subsp. lactis 11454; G, L. lactis subsp. cremoris parental C3; H, L. lactis subsp. cremoris Nip$^+$Suc$^+$ transconjugant NSC3, which contained pGK13; I, CsCl purified pGK13 (37); and J, E. coli V517 size standard plasmids (25). Chr, chromosome.

TABLE 3. Effects of erythromycin, nisin, α-chymotrypsin treatment of cells, and cell washing, on conjugal transfer of Nip$^+$Suc$^+$[a]

Method	Modification	Transconjugants/donor
DPC	None[b]	7.0×10^{-6}
DPC	Nisin (10 ug/ml) included in selective plates.	3.4×10^{-8}
DPC	400 ug of α-chymotrypsin per ml added to mating mixture.	5.2×10^{-6}
DPC	Mating performed between donor and recipient cells which had been harvested from milk agar after 18 h incubation[c].	1.0×10^{-7}
Milk[d]	Alpha-chymotrypsin treatment of Steele and McKay (35).	1.6×10^{-6}
Milk[d]	Alpha-chymotrypsin not included.	6.5×10^{-7}
Milk[d]	Donor washed 3x in 5 ml 0.85% saline before mating then suspended in 1 ml saline. Alpha-chymotrypsin included.	9.1×10^{-6}
Milk[d]	Same as above except no α-chymotrypsin was added.	7.2×10^{-6}

[a] L. lactis subsp. lactis 11454 x L. lactis subsp. lactis LM2306.
[b] Standard DPC mating, for details see text.
[c] Donor and recipient cells were prepared by addition of either 66 ul recipient and 33 ul saline or 33 ul donor and 66 ul saline to milk agar. The plates were incubated 18 h then cells were harvested as described for milk agar matings. DPC was then performed.
[d] Cells were recovered from milk plates in 1-ml saline.

the selective media contained an agent that was bactericidal rather than bacteriostatic, matings were performed on BCP-sucrose agar which contained 10 ug nisin (Aplin and Barrett, Ltd., Wiltshire, UK, 3.7×10^7 IU/g) per ml and 5 ug erythromycin per ml. Transfer of Nip^+Suc^+ was detected on these plates, but at the significantly reduced frequency of 3.4×10^{-8}.

Results from the conjugation methods comparison (Table 2) had indicated that the transfer frequencies obtained by DPC could account for all transconjugants that resulted from milk agar matings. This suggested that conjugation occurred predominantly on selective media rather than on the milk agar plates. To test this possibility, the efficiency with which donor and recipient cells could conjugate on selective media after 18 h of incubation on a milk agar plate was determined. The frequency of Nip^+Suc^+ transfer obtained with a standard milk agar mating was compared to that from a DPC mating made between donor and recipient cells which had been harvested from separate milk agar plates after 18 h of incubation (Table 3). Conjugation between the harvested cells was detected, but the frequency, presented in Table 3, was an order of magnitude lower than that obtained from the standard milk agar matings. Other modified conjugal matings were performed to investigate the use of α-chymotrypsin in Nip^+Suc^+ matings. Results from these matings confirmed

that the protease improved transfer of Nip^+Suc^+ on milk agar (35) but that enhanced transfer of Nip^+Suc^+ could be obtained without α-chymotrypsin if donor cells were rigorously washed in saline prior to mating.

DISCUSSION

The objective of this study was to develop an efficient method to conjugally construct nisin-producing strains of L. lactis subsp. cremoris. Nisin production in these strains would be desirable because many commercially important mesophilic starters are mixed or multiple strains of L. lactis subsp. cremoris (20). Use of nisin-producing L. lactis subsp. lactis starters showed that these organisms effectively controlled some of the bacterial contaminants associated with cheese production (24). Unfortunately, the success of these experiments was limited by frequent defects in the cheese quality. This problem was attributed to nisin activity against the other bacterial cultures in the starter blend and because, alone, the nisin-producing L. lactis subsp. lactis produced an inferior quality cheese (21,24). L. lactis subsp. lactis have been associated with the production of bitter peptides and other off flavors in cheese (20,39). For these reasons, inclusion of a single nisin producer among L. lactis subsp. cremoris starters has been impossible in the past, and the exclusive use of nisin-producing L. lactis

subsp. lactis starters has remained unappealing. These shortcomings should be avoided if Nip$^+$Suc$^+$ were conjugally introduced into commercially proven L. lactis subsp. cremoris starters. Investigators have demonstrated nisin inhibition of Listeria, Clostridium, Staphylococcus, Bacillus, and other undesirable gram-positive bacteria (5,7,21,24). Thus, construction of nisin-producing strains from commercial L. lactis subsp. cremoris starters may lead to enhanced product safety and shelf life without any compromise in product quality.

The data presented in Fig. 2 and 4 demonstrated that Nip$^+$Suc$^+$ was conjugally transferred from L. lactis subsp. lactis 11454 to L. lactis subsp. cremoris recipient strains SW224, JBC3, and JKEB7. Although transfer of Nip$^+$Suc$^+$ into SW224 was achieved by the solid surface milk agar method of conjugation, transfer of this trait into JKEB7 and JBC3 was detected only by the DPC method. Characteristics of DPC indicate this technique offers advantages over previous methods. Aside from the improvement in Nip$^+$Suc$^+$ transfer frequency (Table 2), media and time requirements are reduced compared to either milk agar (27) or filter (16) matings. Additionally, because cells may be concentrated prior to mating, the DPC technique may allow for improved detection of very low frequency conjugal events. High-frequency events were readily detected by the cross-streak DPC assay (Fig.1).

Electroporation can be used to construct recipient strains, for conjugation, from a variety of lactic or other bacteria lacking useful genetic markers (8,10,19,31). This technique was used to transform two L. lactis subsp. cremoris strains with the drug resistance plasmid pGK13 or pGB301 to obtain suitable recipients for the study. Similarly, commercial starters which already possess desirable qualities could be transformed by electroporation with a plasmid encoding a selective marker, and traits such as nisin production, bacteriophage resistance (32), or lactose utilization (27) might then be conjugally introduced. Once transconjugants were obtained, the undesirable plasmid could be eliminated by withdrawing the antibiotic pressure required for plasmid maintenance, followed by selection for drug-sensitive isolates. Plasmid loss could be verified by DNA-DNA hybridizations which utilized the purified plasmid as a probe. The advantage to this approach is that a selective marker required for conjugation could be introduced into potential recipients without resorting to mutagenesis. The latter practice has been commonly used to induce a chromosomal mutation for drug resistance. These mutations may be difficult to reverse and the mutagenic treatment may adversely affect other desirable functions in the cell. Electroporation of cells with a drug resistance plasmid should be less likely to damage desirable cell functions and would rapidly yield

suitable recipient cells, and subsequent removal of the selective marker could be easily confirmed.

Transfer of Nip^+Suc^+ into all L. lactis subsp. cremoris recipients was somewhat surprising because of the extreme sensitivity toward nisin exhibited by L. lactis subsp. cremoris. Hurst (21) has suggested that because L. lactis subsp. lactis and subsp. cremoris are struggling for dominance in the relatively new substrate of "milk in a container", they produce bacteriocins aimed at one another. Thus, L. lactis subsp. cremoris has been reported to be the organism most sensitive to nisin, and L. lactis subsp. lactis is the most vulnerable to diplococcin, a bacteriocin produced by some L. lactis subsp. cremoris strains (21). Despite this, L. lactis subsp. cremoris strains were readily obtained which expressed nisin production and immunity.

The bacteriophage spot assays indicated that L. lactis subsp. lactis NS5406 Nip^+Suc^+ transconjugants expressed Rbs^+. This observation was consistent with other reports (17,29). Although Rbs^+ was not detected among the Nip^+Suc^+ L. lactis subsp. cremoris transconjugants, many commercial strains of L. lactis subsp. cremoris are relatively bacteriophage resistant (20,32). Because the Nip^+Suc^+ L. lactis subsp. cremoris transconjugants generated in the study appeared to retain the recipient phenotype for phage resistance, it seems probable that nisin-producing

transconjugants of commercial strains would also retain this parental property. Another characteristic of the L. lactis subsp. cremoris Nip^+Suc^+ transconjugants was that acquisition of Nip^+Suc^+ did not appear to affect the capability for acid production in milk. These features indicated that Nip^+Suc^+ L. lactis subsp. cremoris transconjugants retained parental characteristics which are important in starter cultures. The characterization studies suggested that blends of mixed and multiple nisin-producing L. lactis subsp. cremoris starters, obtained by conjugal transfer of Nip^+Suc^+ into proven commercial strains, would be suited to commercial applications.

DNA-DNA hybridizations demonstrated that the nisin precursor gene was transferred during conjugation. This result agreed with a recent report by Dodd, et. al. (9). The 8.5 kb EcoRI and 4.2 kb HindIII fragments of the Nip^+Suc^+ transconjugant DNA which hybridized to the oligonucleotide probe were also in close agreement with reported data (6,9,22). Because published sequence data has demonstrated that the nisA gene lies within a 4.2 kb HindIII fragment (6), the hybridization results indicated that at least 4.2 kb and perhaps over 8.5 kb of DNA was transferred with Nip^+Suc^+. Although several investigators have suggested plasmid involvement in nisin production (17,35,40), evidence linking plasmid DNA to nisin production has not been confirmed. The hybridization

performed between the oligonucleotide nisA probe and plasmid DNAs showed the nisA gene was not associated with detectable plasmid DNA in the Nip$^+$Suc$^+$ L. lactis subsp. lactis donor or any of the Nip$^+$Suc$^+$ transconjugants. Although this result does not preclude the existence of a plasmid encoding nisin production that may have been lost during the plasmid isolation technique used in the study, the plasmid hybridization data demonstrated that the 29.1 MDa plasmid of 11454 does not encode the nisA gene. Investigators have previously linked this plasmid to Nip$^+$Suc$^+$ through curing studies (17,35).

The investigations of Nip$^+$Suc$^+$ conjugal transfer (Table 3) provided a few interesting pieces of information. Erythromycin, a bacteriostatic agent and inhibitor of protein synthesis, was used to select against donor cells with DPC. Data for DPC, shown in Tables 2 and 3, strongly indicated that transfer frequency was not impaired by the presence of erythromycin. This result suggested that de novo protein synthesis is not required within the erythromycin-sensitive donor cells for conjugation to proceed. This condition has been demonstrated in E. coli, in which genes on the self-transmissible F plasmid which encode transfer capability are expressed constitutively (42). Table 3 shows that the observed transfer frequencies from matings performed in the presence of nisin and Em were approximately 200 times lower than that observed on media

which contained only erythromycin. This observation suggested that the action of nisin on recipient cells inhibited conjugative transfer of Nip^+Suc^+ to a greater extent than did erythromycin inhibition of donor cells. It is possible that donor cells in the presence of erythromycin were capable of DNA transfer beyond a time which corresponded to that where recipient participation was prevented by nisin in the modified mating plates. This interpretation also implied that de novo protein synthesis in donor cells was not essential for conjugation.

Successful transfer on media which contained nisin indicated that transfer and expression of Nip^+Suc^+ occurred relatively quickly on selective media. This inference was based upon spectrophotometric data which indicated recipient cell lysis occurred approximately 4 h after the addition of 10 ug of nisin per ml (data not shown). Transfer on the nisin-erythromycin plates also suggested that the expression of nisin immunity in transconjugants allowed at least some of these cells to survive despite pre-exposure to the bacteriocin before gene transfer and expression were completed.

Results from the DPC mating performed between donor and recipient cells which had been harvested from milk agar suggested that solid surface milk agar conjugation likely involved gene transfer on both milk agar plates and on the selective media. The demonstrated ability of harvested

cells to conjugate on selective media (Table 3) also suggested that reports of lactococcal conjugation in broth (41), calcium alginate beads (36), and on filters (16) require further investigation to confirm conjugation under the study conditions as opposed to possible subsequent transfer on selective media only.

Data presented in Table 3 also confirmed a report that Nip^+Suc^+ transfer with the milk agar technique was improved if cells were suspended in fresh media and α-chymotrypsin was added to the conjugal mixture (35). Alpha-chymotrypsin is known to inactivate nisin, and it has been employed to protect recipient cells from the bacteriocin during conjugation (35). Inclusion of the protease in DPC matings did not improve the transfer frequency of Nip^+Suc^+. Additionally, results showed that washing cells with saline eliminated the need for α-chymotrypsin in all matings (Table 3).

In conclusion, conjugation has been widely described for a variety of phenotypic traits in lactic acid bacteria (13,23,30). This method of gene transfer has been useful in studies which have investigated the genetics and plasmid biology of these organisms. Conjugation has been applied practically to obtain improved phage resistance in lactococcal strains for the dairy industry (32). Organisms which are genetically improved by conjugation bypass many obstacles associated with the commercial application of

strains which contain recombinant DNA molecules (13). For this reason conjugation will probably continue to be an important means to improve strains for these applications.

Despite this background of study and application, mechanisms of conjugation among gram-positive bacteria, except for the pheromone-induced cell-to-cell adhesion between strains of Enterococcus faecalis (11), remain poorly understood. An improved understanding of conjugal mechanisms would facilitate the scope of genetic manipulations that may be performed by conjugation. Results of this study have suggested that certain aspects of conjugation can be examined through manipulation of the DPC technique.

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CHAPTER IV

PHYSIOLOGICAL CHARACTERISTICS OF NISIN-SUCROSE CONJUGAL TRANSFER AND INTERGENERIC EXCHANGE OF GENES ASSOCIATED WITH NISIN PRODUCTION

ABSTRACT

Physiological parameters which affected transfer of the nisin-sucrose (Nip^+Suc^+) phenotype between Lactococcus lactis subsp. lactis 11454 and LM2306, and intergeneric transfer of these traits among lactic acid bacteria were investigated. Recipients for intergeneric matings were obtained by electroporation with drug resistance plasmid pGK13 or pGB301, or through conjugal introduction of the enterococcal broad-host range plasmid pAMβ1. Results indicated Nip^+Suc^+ exchange within the lactococcal model system was aerotolerant but sensitive to temperature and pH. An investigation of cell clumping in 11454 indicated that, unlike lactose plasmid transfer among other lactococci, cell aggregation in 11454 was not related to the efficiency of Nip^+Suc^+ exchange. Results indicated de novo protein synthesis was necessary in recipient but not donor cells on direct-plate conjugation (DPC) matings. This requirement allowed control over the direction of conjugal exchange when both strains in a mating possessed transmissible elements. DPC matings performed between L.

lactis subsp. *lactis* Nip^+Suc^+ donors and *Streptococcus salivarius* subsp. *thermophilus* recipients which contained pAMβ1 yielded transconjugants which had acquired Suc^+ and nisin immunity but did not produce nisin. DNA-DNA hybridizations, however, demonstrated that the nisin structural gene was present in transconjugants. Finally, a 10 kb *Kpn*I fragment which included the nisin structural gene was inserted into pGK13 but lactococcal electrotransformants which contained the recombinant plasmid did not express any of the traits which have been conjugally linked to nisin biosynthesis in 11454.

INTRODUCTION

Nisin is a peptide bacteriocin synthesized by many strains of *Lactococcus lactis* subsp. *lactis* strains. The protein is bactericidal toward a wide spectrum of gram-positive bacteria (24) and a recent study indicated that some gram-negative organisms may also be affected (48). In many countries of the world, nisin has been employed since the late 1950s to effectively control spore-forming bacteria in processed dairy foods and thus prolong the shelf stability of these products (8,24). Nisin has also been approved by the Food and Drug Administration in the United States for utilization in certain pasteurized cheese spreads (13).

At present, benefits in food preservation derived from nisin have relied upon commercial preparations added directly to processed foods. Analogous applications clearly exist within fermented products if the fermentative microorganisms possess the capability to synthesize nisin. Lipinska (30) pioneered studies which demonstrated the efficacy of nisin-producing starter cultures to control clostridial blowing of rennet-set Edam and Emmental cheeses. Unfortunately, these studies also showed that nisin-producing starter cultures inhibited the other lactococci, lactobacilli, and propionibacteria starters required to manufacture quality cheese. Furthermore, nisin-producing strains of L. lactis subsp. lactis alone were found not to possess all of the traits necessary to produce quality cheese (24,30). Subsequent investigations also revealed that nisin-resistant mutants isolated from the starters which produced quality cheese had lost parental qualities which were essential to quality cheese production (30). Consequently, efforts to develop a starter blend for rennet-set cheeses which included nisin producers and consistently yielded a quality product were not successful. Nevertheless, interest in nisin producing starter cultures has persisted because the bacteriocin is known to inhibit many of the pathogenic and spoilage bacteria which contaminate cheeses (4,7,24,30). The development of gene transfer systems for lactic acid

bacteria has presented modern microbiologists with fresh strategies toward the evolution of nisin-producing starter systems for dairy fermentations.

A number of laboratories have now cloned the nisin precursor gene, nisA, and sequence data for approximately 5.5 kb of associated DNA has been presented (6,10,26). Molecular studies have indicated that genes for nisin biosynthesis are likely encoded on the chromosome of most producer strains (Chapter II, this dissertation,22,43,50). In many Nip^+ strains, the genes appear to lie within a 70 kb conjugative transposon, Tn5301, which also includes genes for sucrose utilization (22,43,53). Reduced bacteriophage sensitivity (Rbs^+) has also been linked to Nip^+Suc^+ transfer (18,39). Despite such intimate molecular scrutiny, neither nisin production nor immunity has been reported from clones. Expression in lactic hosts will at minimum require nisA plus genes which encode nisin immunity and enzyme(s) involved in pronisin maturation. The precise location of genes involved in the latter two functions remain unknown but chromosomal analysis of transconjugants have indicated that they are likely located downstream of nisA within Tn5301 (10). Sequence data have identified two large open reading frames (ORFs) of unknown function downstream of nisA (50).

Gasson (15) first reported conjugal transfer of nisin production and immunity (Nip^+) among strains of L. lactis

subsp. lactis and the simultaneous transfer of sucrose-fermenting ability (Suc^+). Although conjugation has been widely described for a variety of phenotypic traits in lactococci (12), this mechanism of gene transfer remains poorly understood in most gram-positive bacteria. An improved understanding of the physiological requirements and molecular processes involved in lactococcal conjugation would facilitate food-grade strain improvements and may also assist efforts to control dissemination of undesirable genes among pathogenic bacteria.

This laboratory recently reported on direct-plate conjugation (DPC), a new method which facilitated the exchange of the Nip^+Suc^+ phenotype among lactococci (5). This paper presents the results of studies which utilized DPC to investigate physiological characteristics of Nip^+Suc^+ transfer within a model system, L. lactis subsp. lactis strains 11454 x LM2306, and the intergeneric exchange of these genes among lactic acid bacteria.

MATERIALS AND METHODS

Bacterial strains. Strains of bacteria utilized for the study are listed in Table 1. All cultures were stored at 4°C and maintained by biweekly transfers. Lactococci were propagated in M17 broth (52), which contained 0.5% glucose or lactose (M17-G or M17-L), as the sole carbohydrate source. Streptococci were grown in M17-L and lactobacilli or Leuconostoc strains were propagated in MRS

TABLE 1. Bacterial strains and plasmids used in the study

Strain	Relevant phenotype[a]	Description (reference)
Lactococcus lactis subsp. *lactis*:		
ATCC 11454	$Nip^+Suc^+Str^sEm^sClu^+$	Nip^+Suc^+ donor (49).
LM2306	$Nip^-Suc^-Mal^-Em^r$	Plasmid-cured recipient derived from strain C2 (49).
LM2301	$Nip^-Suc^-Mal^+Str^rEm^s$	Plasmid-cured recipient derived from strain C2 (58).
LM0230	$Nip^-Suc^-Mal^+Cm^sEm^s$	Plasmid-cured recipient derived from strain C2 (11).
JK2301β	$Nip^-Suc^-Lac^-Em^rVm^s$	LM2301 transconjugant which acquired pAMβ1 (28).
11454β-1	$Nip^+Suc^+Str^sEm^rClu^+$	Clumping transconjugant of JK2301β x 11454 (this study).
11454β-2	$Nip^+Suc^+Str^sEm^rClu^-$	Non-clumping transconjugant of JK2301β x 11454 (this study).
NSD-1	$Nip^+Suc^+Mal^+Str^rEm^s$	Nip^+Suc^+ transconjugant of 11454 x LM2301 (this study).
NS5406	$Nip^+Suc^+Mal^-Em^r$	Nip^+Suc^+ transconjugant of 11454 x LM2306 (5).
JB100	$Nip^-Suc^-Rbs^-Cm^r$	LM0230 electro-transformed with pJB100 (this study).
NS54100	$Nip^+Suc^+Rbs^+Cm^r$	Nip^+Suc^+ transconjugant of 11454 x JB100 (this study).
Streptococcus salivarius subsp. *thermophilus*:		
S3	$Lac^+Nis^sSuc^-Em^s$	Parental strain, this laboratory.
S313	$Lac^+Nis^sSuc^-Em^r$	S3 electrotransformed with pGK13 (this study).
S3β	$Lac^+Nis^sSuc^-Em^r$	Transconjugant of JK2301β x S3 (this study).
NIS3β	$Lac^+Nis^rSuc^+Em^r$	Suc^+ nisin immune transconjugant of 11454 x S3β (this study).
S4	$Lac^+Nis^sSuc^-Em^s$	Parental strain, this laboratory.
S4β	$Lac^+Nis^sSuc^-Em^r$	Transconjugant of JK2301β x S4 (this study).
NIS4β	$Lac^+Nis^rSuc^+Em^r$	Suc^+ nisin immune transconjugant of 11454 x S4β (this study).
S12	$Lac^+Nis^sSuc^-Em^s$	Parental strain, this laboratory.
S1213	$Lac^+Nis^sSuc^-Em^r$	S12 electrotransformed with pGK13 (this study).
S12β	$Lac^+Nis^sSuc^-Em^r$	Transconjugant of JK2301β x S12 (this study).
NIS12β	$Lac^+Nis^rSuc^+Em^r$	Suc^+ nisin immune transconjugant of 11454 x S12β (this study).
Lactobacillus helveticus:		
ATCC 15009	$Nip^-Suc^-Lac^+Em^s$	Parental strain, this laboratory.
JB15009β	$Nip^-Suc^-Lac^+Em^r$	Transconjugant of JK2301β x 15009 (this study).

(Table 1 continued on next page)

TABLE 1. (continued)

Strain	Relevant phenotype[a]	Description (reference)
Lactobacillus sp:		
ML291	$Nip^-Suc^+Em^s$	Plasmid-cured, maltose negative derivative of strain DB29 (31).
YC301	$Nip^-Suc^+Em^r$	ML291 electro-transformed with pGB301 (this laboratory).
JB291β	$Nip^-Suc^+Em^r$	Transconjugant of JK2301β x ML291 (this study).
Leuconostoc mesenteroides subsp. *dextranicum*:		
181	$Nip^-Suc^+Vm^rEm^s$	Parental strain (41).
JB181-13	$Nip^-Suc^+Vm^rEm^r$	181 electro-transformed with pGK13 (this study).
JB181β	$Nip^-Suc^+Vm^rEm^r$	Transconjugant of JK2301β x 181 (this study).
Leuconostoc mesenteroides subsp. *cremoris*:		
44-4	$Nip^-Vm^rEm^s$	Parental strain (9).
JB44-13	$Nip^-Vm^rEm^r$	44-4 electro-transformed with pGK13 (this study).
JB44-4β	$Nip^-Vm^rEm^r$	Transconjugant of JK2301β x 44-4 (this study).
Escherichia coli:		
JM109	*end*A1, *rec*A1, *hsd*R17, Δ(*lac*-*pro*AB), Amp^sCm^s	Cloning host (Promega Corp., Madison, Wis.)
Plasmids:		
pGK13	Em^rCm^r	Lactococcal shuttle vector (27,51).
pGB301	Em^rCm^r	Streptococcal cloning vector (3).
pGEM-3Z	*lacZ*Amp^r	*E. coli* cloning vector (Promega Corp., Madison, Wis.).
pAMβ1	$Em^rCm^rTra^+$	Enterococcal broad-host range conjugative plasmid (29).

[a]Abbreviations: Nip^+, nisin production and immunity; Suc^+, sucrose utilization; Lac^+, lactose utilization; Mal^+, maltose utilization; Nis^r, nisin immunity; Em^r, erythromycin resistant; Str^r, streptomycin resistant; Cm^r, chloramphenicol resistant; Vm^r, vancomycin resistant; Clu^+, cell aggregation; Rbs^+, reduced bacteriophage sensitivity.

broth (Difco Laboratories, Detroit, Mich.). Lactococci, Leuconostoc spp., and Lactobacillus sp. ML291 derivatives were grown at 30°C. Lactobacillus helveticus and Streptococcus salivarius subsp. thermophilus strains were incubated at 37°C. Escherichia coli were grown in either Brain-Heart Infusion (BBL Beckton Dickinson Microbiology Systems, Cockeysville, Md.) or LB (37) broth at 37°C with aeration.

Electroporation. To obtain suitable recipients for conjugation, lactic acid bacteria were electroporated with either the 4.9 kb plasmid pGK13 (27,51), or the 9.8 kb plasmid pGB301 (3). These plasmids each encode resistance to erythromycin and chloramphenicol which are expressed in lactic hosts. Electroporations were performed with a Bio-Rad (Richmond, Calif.) Gene Pulser™ or a Prototype Design Services Model ZA1000 electroporation unit (Madison, Wis.). Electro-transformation of lactococci was performed with glycine-treated cells as described by Holo and Nes (21) and electroporation of Escherichia coli was by the method of Smith et al. (46). Electro-transformation of other lactic acid bacteria was achieved as reported previously (5). After 24 to 48 h of incubation, plates were examined for antibiotic-resistant colonies and agarose gel electrophoresis was employed to detect transformed plasmid DNA in cell lysates.

Direct-plate conjugal matings. Techniques for standard and cross-streak DPC matings, and subsequent characterization of transconjugant colonies were described previously (5). Indicator agar (36) which contained antibiotic, a single carbohydrate source, and the pH indicator bromo-cresol purple (BCP) was utilized to detect transconjugants. Frequencies for conjugal events were expressed as the number of transconjugants per donor CFU and values reported were the average of at least three separate experiments.

Solid surface milk agar conjugation. Solid surface conjugal matings were performed by the milk agar method of McKay et al. (35). Transconjugants obtained from solid surface matings were harvested and identified as previously described (5). Transfer frequencies were calculated as described for DPC matings.

Environmental influences on Nip^+Suc^+ transfer efficiency. The susceptibility of Nip^+Suc^+ transfer within a model system, L. lactis subsp. lactis ATCC 11454 x L. lactis subsp. lactis LM2306, to temperature, oxygen, and pH was examined. The effect of different temperatures was determined by conjugation between donor and recipient cells grown at either 21°, 30°, or 37°C. To minimize extraneous environmental contributions to the results, selective agar plates were stored at each mating temperature for several hours prior to conjugations. Donor and recipient cells

were grown overnight at each of the three temperatures, inoculated at 1.5% into fresh broth, then incubated again at the original temperature. After 4 h of growth, the A_{600} of each culture was determined and utilized, after cell washes, to calculate a volume of 0.85% saline in which to suspend cells such that all were of approximately the same concentration. When conjugations were performed, only four temperature-equilibrated plates were removed from the incubators at one time, and the four were immediately returned upon addition of cells. The effect of temperature upon conjugation was then inferred from the resultant transfer frequencies.

To determine whether conjugation was aerotolerant, transfer frequencies were compared between simultaneous matings incubated aerobically or in BBL (Beckton Dickinson Microbiology Systems) anaerobic GasPaks. The effect of agar pH upon conjugation was determined by a comparison of frequencies obtained from matings performed on BCP-sucrose which contained 7 ug of erythromycin per ml, and which had been preadjusted to a pH of 5.0, 7.0, or 9.0. After 48 h incubation, pH mating plates were replica-plated onto fresh BCP-sucrose agar (pH 6.9) with erythromycin to confirm the identity of transconjugant CFU.

Effect of protease and amylase treatments to donor cells upon Nip$^+$Suc$^+$ transfer. To investigate whether Clu$^+$ in 11454 was involved in Nip$^+$Suc$^+$ transfer and

biochemically similar to that among high-frequency Lac^+ donors, 11454 was treated with protease and amylase enzymes immediately prior to DPC, as described by Wang and Kondo (59). The proteolytic enzymes utilized were α-chymotrypsin, proteinase K, and pronase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Amylase enzymes (Sigma Chemical Co., St. Louis, Mo.) included α-amylase, beta-amylase, and dextranase. Prior to use, enzymes were dissolved in 0.1 M Na_2HPO_4 buffer, then sterilized by passage through a 0.45 um filter. After cell washes, donor cells were suspended in enzyme solution while recipient and control donor cells were suspended in buffer alone. Cells were then incubated for 15 min before DPC matings were performed. Buffer pH and incubation temperatures were selected as per the enzyme supplier's directions and are provided in Table 3.

Investigation of protein synthesis requirement in recipient cells. To investigate whether recipient cells required de novo protein synthesis for Nip^+Suc^+ transfer, DPC and milk agar conjugations were simultaneously performed between L. lactis subsp. lactis 11454 and JK2301β. JK2301β contained the self-transmissible enterococcal plasmid pAMβ1 (29) which encodes resistance to erythromycin. Because selection of transconjugants was based upon Suc^+ and erythromycin resistance (Em^r), 11454 and JK2301β could each function as donor or recipient. A

1:1 mixture (100 ul final volume) of 11454 and JK2301β was utilized for DPC and milk agar matings. $Suc^{+}Em^{r}$ transconjugants isolated from those conjugations were transferred to M17-L broth which contained 10 ug per ml of nisin and 7 ug per ml of erythromycin. After overnight incubation, Lac^{-} isolates identified by low turbidity in M17-L were transferred to M17-G broth which contained nisin and erythromycin. Plasmid analysis was then performed upon $Lac^{+}Nis^{r}Em^{r}$ and $Lac^{-}Nis^{r}Em^{r}$ isolates to determine whether they represented erythromycin resistant transconjugants of 11454 which received pAMβ1 or JK2301β recipients which had acquired $Nip^{+}Suc^{+}$.

Detection of high-frequency $Nip^{+}Suc^{+}$ donors. Several laboratories have reported lactococcal Lac^{+} transconjugants which subsequently transferred plasmid-borne genes for lactose utilization at very high frequency (17,58,59). To determine whether high-frequency $Nip^{+}Suc^{+}$ exchange could be obtained from $Nip^{+}Suc^{+}$ transconjugants, DPC cross-streak matings (5) were performed between L. lactis subsp. lactis strains 11454 and LM2301 on BCP-sucrose agar which contained 5 mg of streptomycin per ml. $Nip^{+}Suc^{+}$ transconjugants were isolated and then utilized as donors in cross-streak matings with L. lactis subsp. lactis LM2306 on BCP-sucrose agar which contained 7 ug of erythromycin per ml. Strain 11454 was also included and the number of Suc^{+} transconjugants obtained from each donor was compared.

Nip$^+$Suc$^+$ LM2301 transconjugants which produced 10 or more times the number of LM2306 Suc$^+$ transconjugants than had 11454 were subsequently utilized in standard DPC matings with LM2306 to determine actual frequencies of Nip$^+$Suc$^+$ transfer.

Conjugal exchange of pAMß1. To construct some of the recipients for the study, intergeneric conjugal transfer of the broad-host range enterococcal plasmid pAMβ1 was performed between L. lactis subsp. lactis JK2301β and other lactic acid bacteria by the solid surface milk agar technique of McKay et al. (35). Cells on milk plates were incubated anaerobically for 18 h at a temperature (30° or 37°C) which favored recipient cell growth. The cell mixture was then harvested from the agar in 0.6 ml of 0.85% saline, spread upon selective agar, and incubated anaerobically for 48 h to detect transconjugants. Erythromycin resistant transconjugants of S. salivarius subsp. thermophilus and Lactobacillus helveticus 15009 were detected upon BCP-lactose agar which contained 7 ug per ml of erythromycin. Transconjugants from matings between JK2301β and Lactobacillus sp. strain ML291 were isolated from BCP-sucrose agar which contained 7 ug per ml of erythromycin. Leuconostoc spp. transconjugants were detected upon MRS plates which contained 25 ug per ml of vancomycin (41) and 7 ug per ml of erythromycin. Because intergeneric exchange of pAMβ1 has been widely reported

(9,42,56) and was not the focus of this study, transfer frequencies for this plasmid were not calculated.

To determine whether cotransfer of pAMβ1 and Nip^+Suc^+ could be detected, DPC matings were performed between the lactococcal recipient LM2301 and Nip^+Suc^+ donors 11454β-1 and 11454β-2. Conjugations were performed upon BCP-sucrose agar which contained 5 mg per ml of streptomycin. After 48 h of incubation, Suc^+ streptomycin resistant transconjugants were transferred to BCP-sucrose agar which contained 7 ug per ml of erythromycin. All erythromycin resistant isolates were then purified and subjected to plasmid analysis to determine whether pAMβ1 could be detected in lysates of these transconjugants.

Intergeneric conjugations. To determine whether intergeneric exchange of Nip^+Suc^+ could be detected by DPC, Nip^+Suc^+ donors L. lactis subsp. lactis 11454 and NSD-1 were utilized in matings with recipient strains of streptococcus salivarius subsp. thermophilus, Lactobacillus spp., and Leuconostoc spp. Donor and recipient cells for conjugation were prepared as described previously (5). DPC Nip^+Suc^+ conjugations between lactococci and S. salivarius subsp. thermophilus recipients or Lactobacillus helveticus 15009β were performed upon BCP-sucrose agar which contained 7 ug per ml of erythromycin. Plates were incubated anaerobically at 37°C for 72 h.

Intergeneric conjugations between lactococcal Nip^+Suc^+ donors and Lactobacillus sp. strain YC301 and JB291β, Leuconostoc mesenteroides subsp. dextranicum 181β and 181-13, and Leuconostoc mesenteroides subsp. cremoris 44-4β and 44-13, were performed upon milk agar which contained 7 ug per ml of erythromycin. After 18 h of anaerobic incubation at 30°C, cells were harvested from the agar in 1 ml of 0.85% saline and 100 ul aliquots were transferred to MRS broth containing 7 ug per ml each of nisin and erythromycin. MRS broth for cells harvested from Leuconostoc spp. matings also contained 25 ug per ml of vancomycin. Broth tubes were incubated at 30°C for 72 h and examined for turbidity.

DNA isolation and manipulation. Plasmids were isolated by the method of Anderson and McKay (1), and if needed, purified by $CsCl_2$ density gradient centrifugation (33). The presence of plasmids in cell lysates was established by electrophoresis in 0.6% agarose gels at 3 V/cm for 7 h with $CsCl_2$-purified plasmids from E. coli V517 (32) or the BRL supercoiled DNA ladder (Bethesda Research Laboratories, Life Technologies Inc., Gaithersburg, Md.) included for plasmid size standards.

Genomic DNA was isolated by a previously described (5) modification of the Anderson and McKay procedure (1). Restriction analysis of genomic DNA was performed as per the enzyme manufacturer's directions (Bethesda Research

Laboratories or International Biotechnologies Inc., New Haven, Conn.). Prior to electrophoresis, 3 ul of RNase A (Sigma Chemical Co.; 1 mg/ml in 10 mM Tris-HCl, pH 8.0) was added to each sample. Restricted genomic DNA was separated in 0.8% agarose gels at 1.4 V/cm for 18 h. Raoul™ I marker and phage lambda DNA restricted with EcoRI and HindIII were obtained from American Synthesis Inc. (Pleasanton, Calif.) and utilized for fragment size standards.

Cloning of nisA from strain NS5406 was performed in E. coli JM109 with pGEM-3Z (Promega Corp., Madison, Wis.). The vector was linearized with KpnI, dephosphorylated, then mixed with NS5406 KpnI fragments which had been purified from 0.8% agarose with a USBioclean kit (United States Biochemical, Cleveland, Ohio). DNA ligation was performed as described by Maniatis et al. (33). After electroporation, E. coli JM109 cells were plated upon LB agar which contained 0.1 mg ampicillin per ml. Also included on the agar was 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), and isopropyl-β-D-thio galactopyranoside (IPTG) for chromogenic identification of recombinant electro-transformants.

Oligonucleotide nisA probe synthesis and DNA-DNA hybridizations. Synthesis of the 14-mer oligonucleotide probe (5'-ATGTTACAACCCAT-3') utilized to detect the nisA gene in cells, 5'-end labelling with [γ-^{32}P]ATP, DNA-DNA

hybridizations, and autoradiography were described previously (5). DNA was transferred to GeneScreenPlus™ nylon membranes (E.I. du Pont de Nemours & Co., Inc., NEN Products, Boston, Mass.) as described by Southern (47).

Assays. Nisin production by transconjugants was verified by agar overlay as described by Steele and McKay (49). The ability to utilize sucrose was determined by growth and acidification upon BCP-sucrose agar. Reduced phage sensitivity was determined by spot (34) and plaque assays using M17-G agar (52).

RESULTS

Physiological parameters which affected Nip$^+$Suc$^+$ conjugation. DPC matings were utilized to identify physiological parameters which affected Nip$^+$Suc$^+$ transfer in a lactococcal model system. Results from conjugations which investigated the effects of temperature, pH, and aeration are provided in Table 2. Data for different temperatures indicated that at 37°C, Nip$^+$Suc$^+$ exchange occurred at a frequency 2000-fold lower than was detected at 21° or 30°C. Matings performed upon agar of pH 5.0, 7.0, or 9.0 demonstrated that conjugation was favored under neutral pH and that acid conditions produced a 10-fold loss in conjugal efficiency. Nip$^+$Suc$^+$ transfer was not detected at alkaline pH 9.0. Matings performed with anaerobic incubation yielded a mean transfer frequency slightly lower

TABLE 2. Effects of temperature, pH, and oxygen on Nip$^+$Suc$^+$ transfer[a]

Treatment	Transconjugants/donor CFU
Temperature	
21°C	6.9×10^{-6}
30°C	3.4×10^{-6}
37°C	1.6×10^{-9}
Agar pH[b]	
5.0	2.6×10^{-7}
7.0	1.5×10^{-6}
9.0	ND[c] ($<7.7 \times 10^{-9}$)
Aeration:	
anaerobic	1.3×10^{-6}
aerobic	4.2×10^{-6}

[a]L. lactis subsp. lactis 11454 x L. lactis subsp. lactis LM2306.
[b]To detect transconjugants, cells were harvested after 48 h incubation in 1-ml 0.85% saline then plated upon BCP-sucrose agar (pH 6.9) which contained 7 ug of erythromycin per ml.
[c]Transfer was not detected.

than that obtained in the presence of oxygen (Table 2), but transconjugant colonies were detected 18 h earlier. Table 3 presents Nip$^+$Suc$^+$ transfer frequencies obtained from donor cells treated with pronase, proteinase K, α-chymotrypsin, beta-amylase, α-amylase, and dextranase. Similar conjugation frequencies were obtained between all treated and control donor cells.

Protein synthesis requirement in recipient cells. Preliminary characterization of Suc$^+$ erythromycin resistant (Em^r) transconjugants obtained from milk agar matings between 11454 x JK2301β, indicated that of 55

TABLE 3. Effects of protease and amylase treatment upon conjugal transfer of Nip$^+$Suc$^+$[a]

Treatment	Buffer[b] pH	Incubation Temp. (°C)	Transfer Frequency
Protease[c]:			
pronase	pH 7.4	37	4.1×10^{-6}
control		37	3.3×10^{-6}
proteinase K	pH 7.4	37	8.3×10^{-7}
control		37	9.3×10^{-7}
α-chymotrypsin	pH 7.4	30	2.2×10^{-6}
control		30	5.3×10^{-6}
Amylase[d]:			
β-amylase	pH 4.8	20	1.5×10^{-6}
control		20	1.6×10^{-6}
α-amylase	pH 6.9	20	2.4×10^{-6}
control		20	3.0×10^{-6}
dextranase	pH 6.0	37	4.9×10^{-7}
control		37	5.9×10^{-7}

[a] L. lactis subsp. lactis 11454 x L. lactis subsp. lactis LM2306.
[b] 0.1 M Na_2HPO_4
[c] 36 U protease per ml buffer.
[d] 500 U amylase per ml buffer.

colonies characterized, 32 were $Lac^+Em^rNis^r$ (58.2%) and 23 were $Lac^-Em^rNis^r$ (41.8%). Lysates of five Lac^+ isolates all exhibited the plasmid profile of 11454 plus a new plasmid molecule which comigrated with $CsCl_2$-purified pAMβ1 (Appendix Fig. 4). Included among those five isolates were two which no longer exhibited the Clu^+ phenotype of 11454. Plasmid analysis of three Lac^- transconjugants from milk agar matings revealed the plasmid profile of JK2301β. The frequency of Nip^+Suc^+ transfer from 11454 to JK2301β on milk agar was 1.67×10^{-7} transconjugants per donor CFU while pAMβ1 (Em^r) exchange from JK2301β to 11454 occurred at a frequency of 4.91×10^{-8}.

In contrast, analysis of Suc^+Em^r transconjugants obtained from DPC matings revealed that 75 of 75 colonies characterized were $Lac^-Em^rNis^r$, and plasmid analysis of 23 isolates demonstrated that all were Nip^+Suc^+ transconjugants of JK2301β. The frequency of Nip^+Suc^+ exchange from 11454 to JK2301β on DPC was 1.12×10^{-5} transconjugants per donor CFU.

Identification of high-frequency Nip^+Suc^+ donors. DPC cross-streak matings were performed between recipient LM2306 and Nip^+Suc^+ donor strains 11454, and 2 Nip^+Suc^+ transconjugants of LM2301, NSD-1 and NSD-2. After incubation of the mating plates, more than 10 times the number of Suc^+ LM2306 transconjugant colonies were detected from the NSD donor strains as compared to 11454 (Appendix

Fig. 5). Standard DPC matings subsequently showed that 11454 transferred Nip^+Suc^+ to LM2306 at a frequency of 7.73×10^{-6}, while NSD-1 produced a frequency of 1.20×10^{-4} and NSD-2 generated 9.25×10^{-5} Nip^+Suc^+ transconjugants per donor CFU.

Conjugal exchange of pAMß1. Previous studies have demonstrated intergeneric transfer of pAMβ1 among lactic acid bacteria (9,42,56). In this study, putative erythromycin resistant transconjugants were isolated after matings between L. lactis subsp. lactis JK2301β and S. salivarius subsp. thermophilus, Lactobacillus spp., and Leuconostoc spp. parental strains. Cell lysates of transconjugants isolated from each recipient all exhibited the unique plasmid profile of that recipient plus a new molecule which comigrated through 0.6% agarose with $CsCl_2$-purified pAMβ1 (e.g., Appendix Fig. 6).

Analysis of 208 Suc^+Str^r transconjugants isolated from matings between L. lactis subsp. lactis 11454β Nip^+Suc^+ donors and LM2301 identified two erythromycin resistant colonies. Plasmid analysis of these isolates demonstrated that one was a streptomycin resistant mutant of 11454β-1 while the other exhibited a plasmid-cured profile (data not shown).

Intergeneric transfer of nisA. DPC matings for Nip^+Suc^+ exchange performed between L. lactis subsp. lactis 11454 and Streptococcus salivarius subsp. thermophilus

recipients S3β, S4β, and S12β produced $Suc^{+}Em^{r}$ colonies after 60 h of anaerobic incubation at 37°C (Table 4). Characterization of the Suc^{+} transconjugants demonstrated that they were also immune to nisin (Nis^{r}) and had no new detectable plasmid DNA (Appendix Fig. 7). Agar overlay assays for nisin production, however, indicated that these transconjugants did not produce the bacteriocin (Appendix Fig. 8). To determine whether nisA was present, DNA-DNA hybridizations were performed between the oligonucleotide probe and HindIII restricted genomic DNA isolated from a nisin immune Suc^{+} transconjugant, NIS12β, and the recipient strain S12β. Autoradiography located nisA on a 4.2 kb HindIII fragment in the transconjugant but hybridization to the nisin sensitive recipient was not detected (Fig. 1).

Transconjugants were not detected in matings between $Nip^{+}Suc^{+}$ donors and S. salivarius subsp. thermophilus recipients S313 or S1213, Lactobacillus helveticus 15009β, Lactobacillus sp. strains YC301 or JB291β, Leuconostoc mesenteroides subsp. dextranicum 181β or 181-13, or Leuconostoc mesenteroides subsp. cremoris 44-4β or 44-13.

Construction of pJB100. DNA-DNA hybridization between the oligonucleotide nisA probe and KpnI restriction endonuclease digests of NS5406 chromosomal DNA identified a 10 kb fragment which contained nisA (Fig. 2a). Examination of published sequence data indicated that the 5'end of the KpnI fragment likely was located within the putative transposase gene of IS904 which is upstream of nisA (6,10).

TABLE 4. Frequencies for intergeneric transfer of nisin-sucrose genes[a]

Recipient	Transfer Frequency
Streptococcus salivarius subsp. *thermophilus*	
S3β	2.1×10^{-9}
S4β	2.0×10^{-9}
S12β	2.3×10^{-9}
S313	N.D.[b] ($<1.0 \times 10^{-9}$)
S1213	N.D.[b] ($<1.0 \times 10^{-9}$)
Lactobacillus helveticus	
15009β	N.D.[b] ($<1.1 \times 10^{-9}$)

[a]From matings where frequency calculations were possible (see Chapter IV methods), the donor strain was *L. lactis* subsp. *lactis* 11454. Transfer included genes for nisin immunity, sucrose utilization, and the nisin structural gene *nisA*.
[b]Transfer was not detected.

To clone the fragment, *Kpn*I fragments of NS5406 which approximated 10 kb were purified from 0.8% agarose gels, ligated into pGEM-3Z, and electro-transformed into *E. coli* JM109. Plasmid analysis of Lac$^-$, ampicillin-resistant *E. coli* electro-transformants identified several putative recombinant molecules. When agar overlay assays did not detect nisin production from the putative recombinants, DNA-DNA hybridizations were performed between the *nisA* probe and *Kpn*I restricted plasmid DNA isolated from the clones. Autoradiography identified two recombinant molecules which contained *nisA* (Appendix Fig. 9). To determine the orientation of fragment insertion, both clones were digested with *Pst*I. This enzyme cut pGEM-3Z

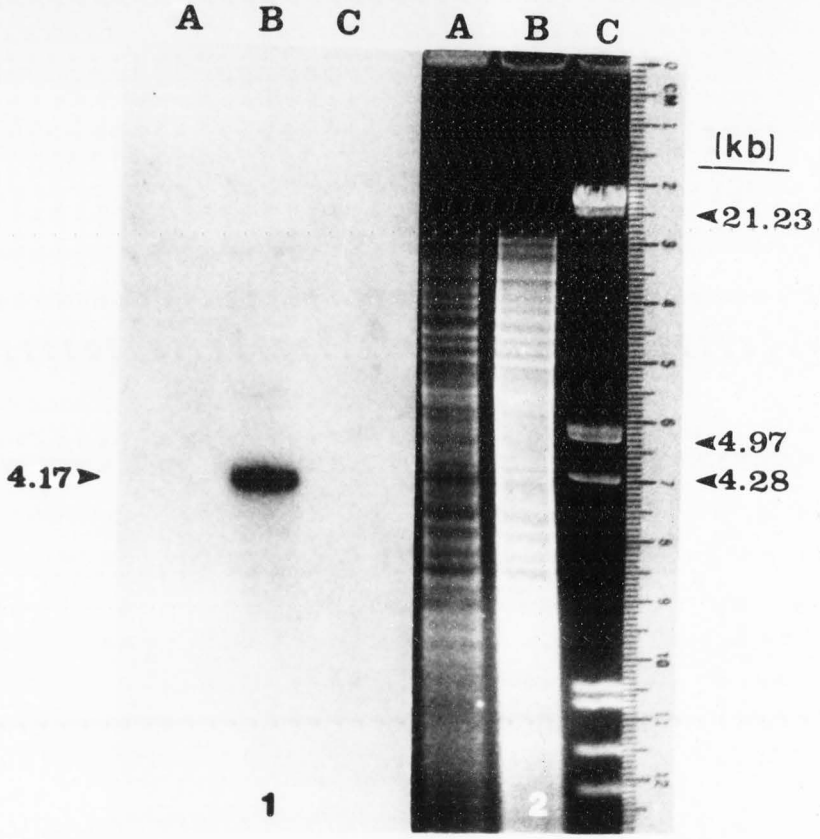
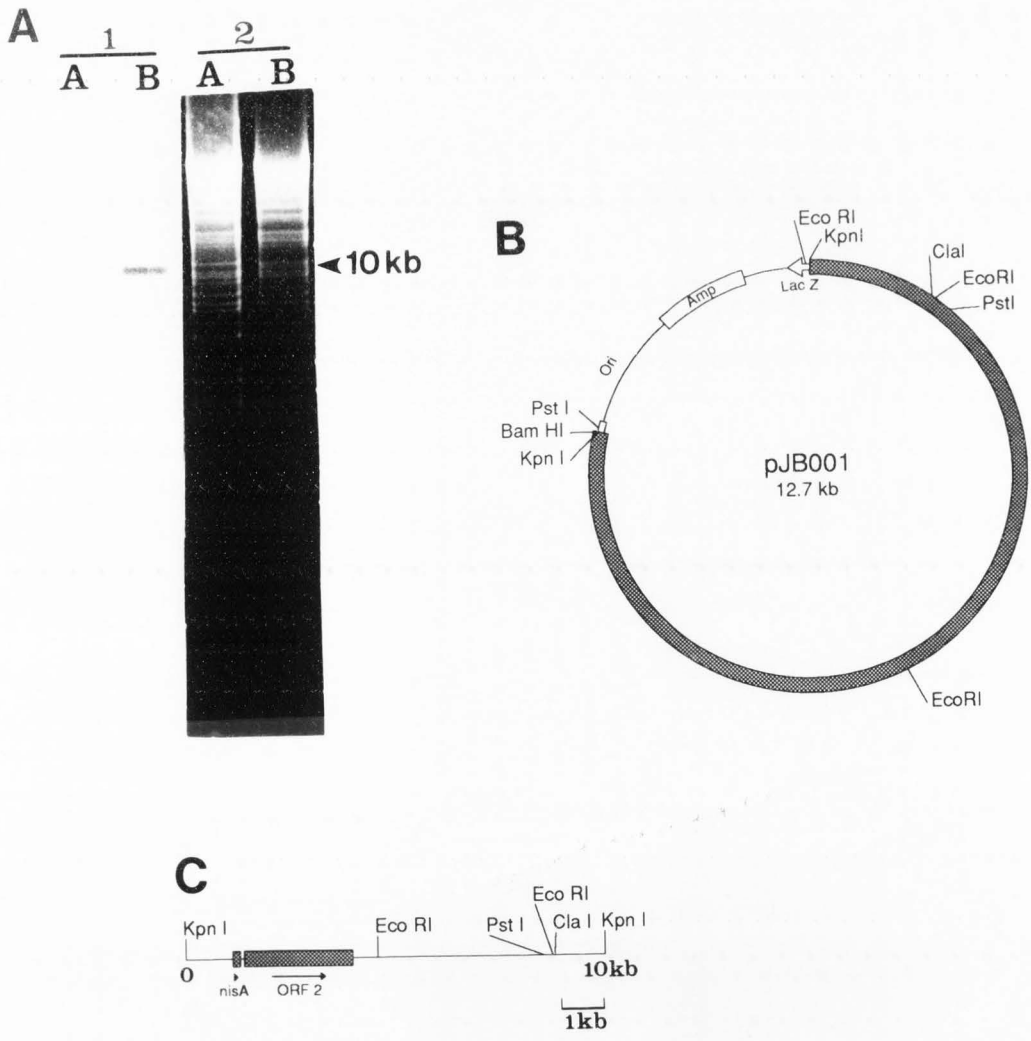


FIG. 1. Hybridization of HindIII restricted genomic DNA from S. salivarius subsp. thermophilus recipient S12β and its' Suc$^+$ transconjugant, NIS12β, by an oligo nisA-specific probe. Panel 1 shows the autoradiogram obtained after exposure of the blot, made from the agarose gel shown in panel 2, which was probed with the 5'-end labelled oligonucleotide. Hybridization was performed at room temperature for 24 h as described previously (5). DNA samples which corresponded to the lanes in each part were; A) HindIII digested S12β, B) HindIII digested NIS12β, and C) EcoRI and HindIII digested bacteriophage lambda DNA size standard fragments.

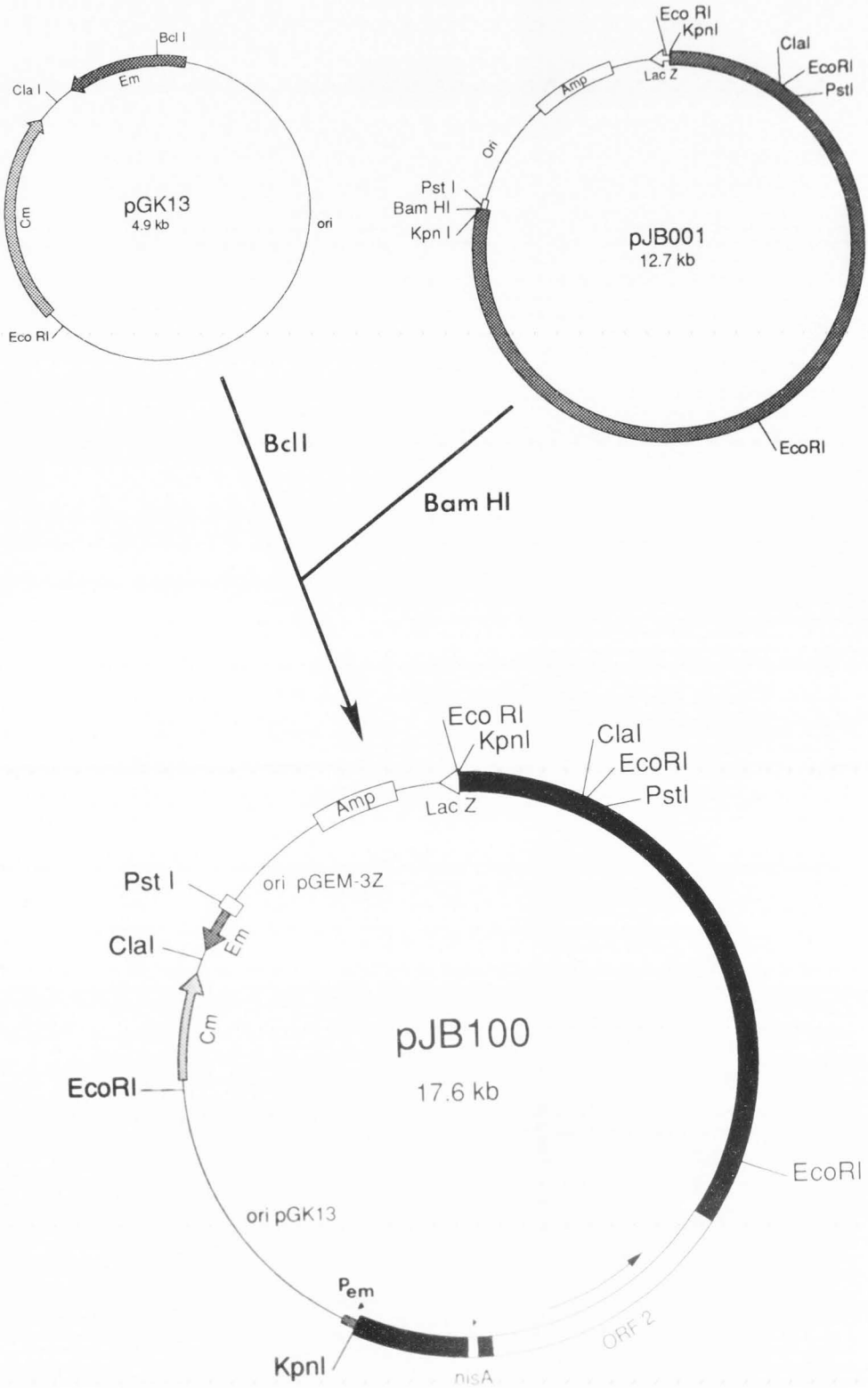
FIG. 2. Cloning the nisA gene from L. lactis subsp. lactis NS5406. Part A involved hybridization of the oligonucleotide nisA probe to KpnI restricted chromosomal DNA from L. lactis subsp. lactis recipient LM2306 and its Nip^+Suc^+ transconjugant, NS5406. Panel 1 shows the autoradiogram obtained after exposure of the blot, made from the agarose gel shown in panel 2. Hybridization was performed at room temperature for 24 h as described previously (5). Chromosomal DNA samples which corresponded to the lanes in each part were; A) KpnI digested LM2306 and B) KpnI digested NS5406. Results identified a 10 kb KpnI fragment in NS5406 which contained nisA. Part B shows recombinant plasmid pJB001. NS5406 KpnI fragments which approximated 10 kb were purified from 0.8% agarose as described in the text and ligated into the unique KpnI site in the pGEM-3Z polylinker region of lacZ. A restriction endonuclease map of pJB001 was constructed and compared to published DNA sequence and map data for nisA (6,10,50). The comparison yielded the map, shown in part C, which included the location of nisA and a downstream open reading frame (ORF-2). Arrows have been added to denote the orientation of the open reading frames.


A
1
2
A B A B
10kb
B
Eco RI
KpnI
ClaI
EcoRI
PstI
Amp
Lac Z
Ori
Pst I
Bam HI
Kpn I
pJB001
12.7 kb
EcoRI
C
Kpn I
Eco RI
Pst I
Eco RI
Cla I
Kpn I
0
10kb
nisA
ORF 2
1kb

only once and had previously been mapped approximately 6 kb downstream of nisA in 11454 (50). Results demonstrated that both orientations had been obtained and the one which placed nisA immediately downstream of the pGEM-3Z lacZ promoter was designated pJB001 (Fig. 2b). To determine whether lactococci which contained the nisA fragment would express traits previously linked to nisin biosynthesis in 11454 (18,49), pJB100 was constructed. For this construct, pJB001 was linearized with BamHI then ligated into the unique BclI site within the erythromycin resistance gene on pGK13 (Fig. 3). The ligation mix was electroporated into E. coli JM109 and electro-transformants were isolated from LB agar which contained 100 ug per ml of ampicillin and 20 ug per ml of chloramphenicol. Plasmid DNA isolated from ampicillin and chloramphenicol resistant transformants was restricted with EcoRI to determine the orientation of pJB001 insertion into pGK13. The orientation which placed nisA approximately 1.4 kb downstream of the pGK13 erythromycin resistance gene promoter was designated pJB100 (Fig. 3), while the opposite orientation was denoted as pJB050.

Plasmids pJB100 and pJB050 were then purified through $CsCl_2$ density gradients and electroporations were performed with L. lactis subsp. lactis LM0230. Plasmid analysis of putative transformants isolated from LM0230 cells electro-transformed with pJB100 demonstrated the presence of a new molecule which migrated through 0.6% agarose with $CsCl_2$-

FIG. 3. Construction of pJB100. To determine whether lactococci which carried the 10 kb KpnI nisA fragment would express traits which had been associated with nisin biosynthesis in 11454 (18,49), pJB001 was opened at the unique BamHI site located in the pGEM-3Z polylinker, then ligated into the unique BclI site of pGK13. Genetic analysis of ampicillin and chloramphenicol resistant E. coli JM109 electro-transformants identified clones which contained pJB100. This molecule placed nisA approximately 1.4 kb downstream of the erythromycin resistance gene promoter (P_{em}) of pGK13. Chloramphenicol resistant electro-transformants of L. lactis subsp. lactis LM0230 which contained pJB100 were isolated and then assayed for nisin production and immunity, sucrose utilization, and reduced bacteriophage sensitivity as described in the text. Arrows denote the direction of gene expression as determined by published DNA sequence data (6,10,27,50).


Bcl I
Em
Cla I
Cm
pGK13
4.9 kb
ori
Eco RI
Eco RI
KpnI
ClaI
EcoRI
PstI
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Lac Z
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Eco RI
KpnI
ClaI
EcoRI
PstI
Amp
Lac Z
Pst I
ori pGEM-3Z
ClaI
Em
Cm
EcoRI
pJB100
17.6 kb
EcoRI
ori pGK13
Pem
KpnI
nisA
ORF 2

purified pJB100. Transformants were not detected among cells electroporated with pJB050. When transformant JB100 was assayed for nisin production or immunity, sucrose utilization, and reduced phage sensitivity (Rbs^+), none of the traits were detected.

Conjugations were then performed between 11454 and JB100 to determine if Nip^+Suc^+ transconjugants which contained pJB100 would produce a larger zone of inhibition from the agar overlay Nip^+ assay. A single transconjugant, NS54100, was isolated and characterized (Table 1). Nip^+ assays revealed similar zones of indicator strain inhibition from L. lactis subsp. lactis NS54100, NS5406, and 11454 (data not shown).

DISCUSSION

The objective of this study was to identify physiological parameters which affected Nip^+Suc^+ transfer and to investigate intergeneric exchange of these genes among lactic acid bacteria. This information would facilitate the development of nisin-producing starter cultures for dairy fermentations. Nisin production in dairy lactic acid bacteria would be desirable because prior use of nisin-producing L. lactis subsp. lactis starters demonstrated that these organisms effectively controlled clostridial spoilage in rennet set Edam and Emmental cheese (30). Unfortunately, nisin also inhibited desirable lactic

acid bacteria so inclusion of nisin producers in mixed starter cultures was not possible and the nisin-producing L. lactis subsp. lactis alone yielded an inferior quality cheese (24,30). Despite these limitations, interest in nisin-producing starter blends has persisted because nisin has been shown to inhibit species of Listeria, Clostridium, Staphylococcus, Bacillus, and other gram-positive bacteria which occasionally contaminate cheese (4,7,24,30). Thus, development of nisin-producing starter cultures may provide an avenue toward enhanced product safety and shelf life without compromise to product quality.

Although nisin resistance genes have been isolated from lactic acid bacteria (14,57), utilization of these genes in dairy starter cultures may not be advantageous if nisin is desired in the fermented product. This is because some of these genes may encode nisinase or similar enzymes which degrade or remove nisin from the growth medium. Roberts et al. (44) reported this phenomenon among L. lactis subsp. lactis C2 transformed with a nisin resistance gene originally isolated from L. lactis subsp. lactis biovar. diacetylactis. An alternative and viable method to develop nisin-producing dairy starter cultures is conjugation. This form of gene transfer offers advantages even over recombinant DNA technology when food applications are involved (45). An improved understanding of the molecular and physiological processes involved in

conjugation would facilitate dairy strain improvements and may also help to prevent the dissemination of undesirable genes among pathogenic bacteria.

For these reasons, direct-plate conjugation was employed to investigate parameters which affected Nip^+Suc^+ transfer between L. lactis subsp. lactis 11454 and LM2306. Data presented in Table 2 indicated that Nip^+Suc^+ transfer was temperature sensitive. Only 18 transconjugant colonies were detected from a total of 18 conjugations performed at 37°C. The effect was not attributed to decreased cell viability since the A_{600} of 4 h cultures grown at 21°C was consistently lower than that of cells propagated at 37°C. Although conjugation between lactococci was strongly inhibited at 37°C, intergeneric transfer of Suc^+ and nisin immunity to S. salivarius subsp. thermophilus recipients S3β, S4β, and S12β was detected on plates incubated at 37°C. The lactococcal donors for these matings were grown at 30°C. Conjugation between Nip^+Suc^+ lactococci and streptococcal recipients occurred with similar efficiency if the recipients were grown at 30°C before conjugation. These results suggested that conjugation was inhibited at growth temperatures near the upper limit, while suboptimal growth temperatures had little or no effect upon Nip^+Suc^+ transfer efficiency.

Whitaker and Batt (60) have reported that a 5 to 10°C increase over optimal growth temperature will activate a

heat shock response in lactococci. Studies of heat shock response in E. coli have indicated that cellular functions within the normal temperature range are largely coordinated by modifications in enzyme activity whereas growth at high temperature is accompanied by changes in the content of individual proteins and other cellular components (40). Consequently, growth of lactococci at elevated temperature may deleteriously affect the activity and/or the cellular content of proteins involved in conjugal exchange.

Table 2 presents results from matings performed upon agar of different pH. The frequency of Nip^+Suc^+ conjugal exchange on agar of pH 5.0 was similar to that detected previously from donor and recipient cells incubated separately for 18 h on milk agar then harvested for conjugation (5). The resemblance between transfer frequencies provided physiological evidence to suggest that acid inhibition produced the log decrease in Nip^+Suc^+ conjugal efficiency observed previously. Although L. lactis subsp. lactis is reported to grow at pH 9.0 (38), conjugation was not detected on the alkaline mating plates.

Conjugal frequencies of matings performed under aerobic and anaerobic conditions were relatively similar (Table 2). When plates were incubated anaerobically, however, transconjugants were detected 18 h earlier. The observation that transconjugant CFU were be detected within 24 h supported a previous hypothesis that conjugal exchange

occurred rapidly upon selective media (5). Alternatively, colonies may simply form more rapidly under anaerobic conditions. Most significantly, anaerobic incubation of DPC plates provided conjugal results 36 to 48 h earlier than previously available from solid surface milk agar conjugation techniques (35).

Several laboratories have associated donor cell aggregation (Clu^+) with high-frequency conjugal transfer of Lac^+ in lactococci (17,58,59). Subsequent studies have indicated that cell aggregation is the result of at least two gene products designated Clu and Agg (54). Wang and Kondo (59) recently demonstrated that Clu^+ and high-frequency Lac^+ transfer was sensitive to protease but not amylase treatment on DPC. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was then utilized to correlate those results to a large extracellular protein on Clu^+ cells. Similar enzymatic treatments to 11454 failed to affect Nip^+Suc^+ conjugal efficiency (Table 3) or to disrupt the Clu^+ phenotype in 11454. These results indicated that the aggregation substance in 11454 was distinct from that found among high-frequency Lac^+ donors. Additional evidence to support this hypothesis was obtained from matings between LM2306 and Nip^+Suc^+ donors 11454β-1 and 11454β-2. The efficiency of Nip^+Suc^+ transfer detected from the Clu^+ donor 11454β-1 was actually slightly lower than that obtained for the Clu^- donor 11454β-2; 3.55×10^{-6}

versus 7.95 x 10^{-6} transconjugants per donor CFU. Finally, Clu^+ was not observed among the Nip^+Suc^+ donors NSD-1 or NSD-2. These data suggested that Clu^+ in 11454 was not required for efficient Nip^+Suc^+ transfer and differed both biochemically and functionally from that associated with high-frequency lactose plasmid transfer.

Natural transformation systems have not been demonstrated among lactococci, yet conjugation experiments have reported that DNase treated transformation controls yielded a slight decrease in conjugal frequency (5,35). To investigate whether these data represented an artifact that resulted from the treatment, analogous experiments were performed with RNase A, nuclease S1, and the restriction endonuclease MspI. Results of these matings demonstrated that none of these enzymes produced a reduction in the frequency of Nip^+Suc^+ transfer (Appendix Table 1), which indicated that the original observations were likely artifact.

This laboratory previously reported that Nip^+Suc^+ conjugal transfer did not require de novo protein synthesis within donor cells (5). This conclusion suggested that genes which encode transfer capability in L. lactis Nip^+ donors, like those of the E. coli F plasmid (61), were expressed constitutively. Conversely, recipients should require protein synthesis to express acquired DNA and matings between Suc^+Em^s 11454 and Suc^-Em^r JK2301β on milk

agar and DPC supported this conclusion. Transconjugants from these matings were detected by their ability to utilize sucrose (Suc^+) and grow in the presence of erythromycin (Em^r). Each of these markers was transmissible and present in only one of the cell types. Thus, either strain could function as donor or recipient, based upon which direction gene exchange occurred. If de novo protein synthesis were required in recipient cells, then transfer should occur only toward recipients able to satisfy this requirement. On milk agar both cells were able to synthesize new protein, but on DPC only JK2301β satisfied this requirement. Consequently, bidirectional transfer was detected on the former medium but only unidirectional transfer was obtained on the latter.

Although not unexpected, the de novo protein synthesis requirement of recipients was useful because, on DPC, it permitted control over the direction of genetic exchange when conjugal markers were present in both mating cells. The utility of this feature was demonstrated by the transfer of genes associated with nisin production to S. salivarius subsp. thermophilus recipients which contained pAMβ1. Although the frequency of intergeneric transfer was very low (Table 4), all Suc^+ erythromycin resistant CFU detected were streptococcal transconjugants.

Unpublished data from this laboratory indicated that Nip^+Suc^+ transconjugants were commonly able to donate these

genes in secondary matings. Because Lac$^+$ transconjugants with substantially enhanced transfer capability have been recognized among lactococci (2,17,58,59), we questioned whether a similar phenomenon occurred among Nip$^+$Suc$^+$ transconjugants. Cross-streak DPC between Nip$^+$Suc$^+$ LM2301 transconjugants and LM2306 identified transconjugant donors which appeared to exhibit higher Nip$^+$Suc$^+$ transfer capability than 11454. Standard DPC matings subsequently demonstrated that NSD donors transferred Nip$^+$Suc$^+$ to LM2306 at frequencies 10- to 15-fold greater than did donor strain 11454. Although the frequency increase was considerably lower than the 10^3 to 10^5 enhancement detected from Lac$^+$ transconjugants, enhanced Nip$^+$Suc$^+$ transfer frequency was also detected in intergeneric conjugations. Matings between L. lactis subsp. lactis NSD-1 and S. salivarius subsp. thermophilus S12β indicated that nisin gene transfer was obtained at a frequency of 1.2×10^{-8}. This frequency was approximately 10-fold higher than had been obtained with 11454 (Table 4). Enhanced Nip$^+$Suc$^+$ transfer in both intraspecific and intergeneric conjugations indicated that NSD donors may be useful for the development of nisin-producing dairy starter blends. Because these donors also are Lac$^-$, strain constructions by the technique of Sanders et al. (45) which did not employ antibiotic resistance for transconjugant selection may be possible.

Recipients employed in intergeneric conjugal experiments were included in Table 1. Although intergeneric exchange of pAMβ1 was detected with parental strains of S. salivarius subsp. thermophilus, Lactobacillus spp., and Leuconostoc spp., intergeneric transfer of genes associated with nisin production was demonstrated only with S. salivarius subsp. thermophilus recipients. Transfer to this species was further limited to derivatives which contained pAMβ1, a feature which suggested this plasmid was involved in the intergeneric exchange. One possibility was that pAMβ1 contributed gene products which facilitated conjugal contact between lactococcal and streptococcal cells, but the absence of comparable results among other lactic recipients clouded any conclusions. Although pAMβ1 may have facilitated transfer of nisin genes to streptococci, cotransfer of pAMβ1 and Nip^+Suc^+ was not detected from conjugations between L. lactis subsp. lactis LM2301 and Nip^+Suc^+ donors 11454β-1 and 11454β-2. Other investigators have demonstrated conjugal cotransfer of pAMβ1 and lactococcal plasmid DNA (2,20,55).

Phenotypic analysis of S. salivarius subsp. thermophilus transconjugants indicated that genes for sucrose metabolism and nisin immunity had been transferred from Nip^+Suc^+ Lactococcus lactis subsp. lactis to Streptococcus salivarius subsp. thermophilus recipients, but nisin production was not detected. DNA-DNA

hybridizations between the nisA probe and genomic DNA isolated from a transconjugant, however, demonstrated that nisA also had been transferred. The nisin precursor gene was located in the nisin immune Suc^+ transconjugant, NIS12β, upon a 4.2 kb HindIII fragment which was absent in the recipient (Fig. 1). The 4.2 kb HindIII fragment agreed closely with published sequence data for 11454 and other Nip^+ strains (6,10). Gasson has reported that nisin immune transconjugants of Lactobacillus plantarum also did not produce the peptide (16). Although some lactic transconjugants may not produce nisin, applications likely exist for nisin immune cultures in mixed starter systems which include nisin producers.

Recent reports have indicated that, in many Nip^+ strains, genes for nisin biosynthesis and sucrose utilization lie within a 70 kb chromosomally located conjugative transposon, Tn5301 (22,43). An additional report indicated that nisA and sucrose genes were located upon separate EcoRI fragments in 11454 (53). Because phenotypic and genetic analysis of S. salivarius subsp. thermophilus demonstrated acquisition of nisA, nisin immunity, and sucrose utilization, there was no evidence to suggest that 11454 had not transferred Tn5301 in its entirety to these recipients. The absence of nisin production among these transconjugants indicated that perhaps Tn5301 did not encode all of the genes required for

full expression of Nip$^+$ or else the streptococci could not express some of the lactococcal genes which were acquired. Previous studies of nisin production in lactococci have yielded evidence which may support the former possibility. Those studies identified basic peptides, in Nip$^-$ lactococci, which were electrophoretically similar to nisin (23) and that extracts of Nip$^-$ cells were able to catalyze the conversion of pronisin into active nisin (25). These observations imply that enzyme(s) which convert pronisin to nisin may not be included within Tn*5301* and may in fact have other functions in the cell. Hurst (23) suggested that nisin-like basic peptides were important for regulation of the *L. lactis* subsp. *lactis* growth cycle.

Because *nisA* is expressed constitutively in Nip$^+$ cells, yet active nisin is not detected until mid log phase growth (6,24), we questioned whether pronisin was involved in nisin immunity. To investigate this possibility, a 10 kb *Kpn*I fragment which included *nisA* was cloned from the lactococcal transconjugant NS5406. A restriction map of the construct, JB001, was generated and compared to published sequence and restriction data for *nisA* (6,10,50). The results indicated that *nisA* was located approximately 1.2 kb downstream of the 5' end of the *Kpn*I fragment (Fig. 2c). Steen et al. (50) demonstrated that *nisA* was part of a polycistronic operon in 11454 which included a large downstream ORF. Sequence data further indicated that the

nearest upstream RNA polymerase promoter consensus sequence was associated with the putative transposase gene of IS*904* approximately 1.7 kb from *nisA* (6,10). Because the 5' *Kpn*I site of the cloned fragment was located downstream of the transposase promoter, pJB100 was constructed to provide *nisA* with an RNA polymerase promoter sequence. As shown in Figure 3, pJB100 placed *nisA* approximately 1.4 kb downstream of the erythromycin resistance gene promoter of pGK13 (27). Hardy et al. (19) previously demonstrated that a similar construct promoted expression of heterologous genes in *Bacillus subtilus*. Phenotypic characterization of *L. lactis* subsp. *lactis* LM0230 pJB100 transformants, however, failed to detect nisin immunity or production, sucrose utilization, or reduced phage sensitivity (18,39,49). Interestingly, electro-transformation of pJB050 into LM0230 was not detected even though the plasmid could be readily introduced into competent *E. coli*. This result suggested that pJB050 may have encoded a product(s) that, without expression of *nisA* and/or the cotranscribed ORF immediately downstream (50), was lethal to lactococcal cells.

In conclusion, conjugation has been described for a variety of phenotypic traits in lactococci (12), and mechanisms for intergeneric exchange among lactic acid bacteria have been demonstrated (9,42,56). Conjugation facilitated early studies of genetics and plasmid biology

in lactic acid bacteria and the technique has been utilized to improve bacteriophage resistance in dairy lactococci (45). Food grade microorganisms which are genetically improved by conjugation bypass numerous obstacles which face commercial application of strains which contain recombinant DNA molecules (12,45). For this reason conjugation will likely continue to be an important method for industrial strain improvements. One potential application involves the development of nisin-producing dairy starter cultures. This study identified Nip^+Suc^+ donors which exhibited enhanced transfer capability in intraspecific and intergeneric matings and demonstrated transfer of these genes S. salivarius subsp. thermophilus recipients. Results also indicated that efficiency of Nip^+Suc^+ transfer was sensitive to temperature and pH but, unlike high-frequency lactose plasmid transfer, was not dependent upon donor cell aggregation. Further studies of lactic conjugal mechanisms would facilitate genetic improvements of dairy starter cultures and may also provide information to control the dissemination of undesirable genes.

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CHAPTER V

NISIN INHIBITS SEVERAL GRAM-POSITIVE, MASTITIS-CAUSING PATHOGENS[2]

ABSTRACT

Organisms known to cause bovine mastitis, Enterococcus faecalis subsp. liquefaciens ATCC 27959, Staphylococcus aureus ATCC 29740, Streptococcus agalactiae ATCC 27956, Streptococcus equinus ATCC 27960, Streptococcus dysgalactiae ATCC 27957, Streptococcus uberis ATCC 27958, together with the neotype Staphylococcus epidermidis ATCC 14990, were examined for their susceptibility to the small peptide bacteriocin, nisin. Using a disc assay, minimum inhibitory concentrations of nisin ranged from 10 to 250 ug per ml among the strains. Examination of the antimicrobial effect of 50 ug of nisin per ml in milk showed nisin inhibited all gram-positive pathogens tested.

INTRODUCTION

Mastitis is one of the most widespread and costly diseases affecting dairy herds (2). Morse (13) reported that nearly 50% of cows suffer at least one outbreak of clinical mastitis per lactation. Control of the disease

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involves hygienic practices such as teat dipping and infusion of antibiotic drugs into the udder. Gilmore (6) estimated 33 million antibiotic treatments are given each year in the United States.

Once present, antibiotics cannot be removed from milk (10), so milk from treated cows cannot be sold for 3 to 5 d after treatment. The cost of discarding antibiotic-containing milk is significant (14), but the procedure is necessary to protect the estimated 5-10% of adult Americans who show hypersensitivity to antibiotic drugs (5) as well as starter cultures used in milk processing. Various on-farm screening tests have been developed to determine when milk is free of antibiotics, adding to overhead costs. More significantly, these tests occasionally yield false positive or false negative results (16).

Nisin is a short peptide bacteriocin produced by some strains of Lactococcus lactis ssp lactis. The protein exerts a bactericidal effect on many gram-positive organisms but is not effective against gram-negative bacteria. Nisin is non-toxic to humans and is readily broken down by digestive enzymes when consumed (8). Hypersensitivity to nisin has not been recorded. It has been used as a food preservative in other countries since 1954 (9) and has recently gained approval in the United States for use in certain dairy products (4). These features make nisin a good candidate for mastitis research,

since many mastitic infections involve gram-positive pathogens (3) and nisin-containing milk does not present a threat to consumer health.

The objective of this study was to determine if several species of mastitis-causing pathogens were susceptible to nisin *in vitro*.

MATERIALS AND METHODS

Bacterial Strains. *Enterococcus faecalis* subsp *liquefaciens* ATCC 27959, *Staphylococcus aureus* ATCC 29740, *Staphylococcus epidermidis* ATCC 14990, *Streptococcus agalactiae* ATCC 27956, *Streptococcus equinus* ATCC 27960, *Streptococcus dysgalactiae* ATCC 27957, and *Streptococcus uberis* ATCC 27958 were obtained from American Type Culture Collection (Rockville, Md.). All ATCC cultures used, except the neotype strain of *Staph. epidermidis*, originated from bovine udder infections. *Escherichia coli* V517 (12) was received from Larry McKay (University of Minnesota). The staphylococci were grown in nutrient broth (5% peptone, 3% beef extract, pH 6.8) supplemented with .5% yeast extract. All other strains were kept in Brain Heart Infusion (BHI) broth (BBL, Cockeysville, Md.) supplemented with .5% yeast extract. Cultures were maintained by biweekly transfers, grown at 37°C and stored at 4°C.

Disc Assays. The disc assays were performed with the following modifications to the method of Barry and

Thornsberry (1); a stock nisin (Aplin and Barrett Ltd., Wiltshire, UK, 3.7 x 10^7 IU/g) solution of 10 mg/ml was prepared in double deionized water. Test solutions contained 0, 10, 50, 100, 250, 500, 750, 1000, 2500, 5000, 7500 and 10,000 ug of nisin per ml. All solutions were sterilized using a .45 um syringe-mounted filter. Fresh nisin solutions were prepared immediately before use. Midlog phase cells were obtained by making a 1% inoculation into fresh media from an overnight culture. Cells were then incubated at 37°C until absorbance at 600 nm reached .5 to .7. Optical densities were measured using a Bausch and Lomb Spectronic 20. Disc assays were done on BHI media with .5% yeast extract. Cells were spread onto the media with sterile cotton applicators and the plates were allowed to dry for 2 to 3 min. Thirteen mm filter paper discs (Difco, Detroit, Mich.) were then dipped into the appropriate nisin solution until saturated, touched lightly to the container wall to remove excess fluid, and immediately placed upon the inoculated plate. After 24 h incubation at 37°C, zones of inhibition were measured to the nearest millimeter. The minimum inhibitory concentration (MIC) was the lowest nisin concentration showing a 13.5 mm zone of inhibition to the organism. All experiments were performed in duplicate.

Assays In Milk. Activity of nisin in milk was tested on Escherichia coli V517, Staphylococcus aureus ATCC 29740,

Staphylococcus epidermidis ATCC 14990, Streptococcus agalactiae ATCC 27956, and Streptococcus dysgalactiae ATCC 27957. Cultures were standardized to an absorbance of .5 to .7 as described for the disc assay. For each strain four milk tubes were used, two control and two containing 50 ug of sterile nisin per ml solution. Milk tubes contained 10 ml of 11% non-fat dry milk that was sterilized by steaming for 45 min then heating to 245°F at 12 psi for 12 min. At time zero, 1% inoculations were made into milk tubes and cell counts of each strain were performed by plating onto BHI plus .5% yeast extract. Plates and milk tubes were incubated at 37°C for 24 h and cell counts made at 0, 6, and 24 h. Plate counts were performed in duplicate.

RESULTS AND DISCUSSION

Disk assays. Results of the disc assay are shown in Table 1. All species examined except E. coli, the negative control, were inhibited by nisin. Susceptibilities ranged from 10 to 250 ug/ml. As has been observed previously (9), E. faecalis was the most resistant gram-positive organism tested. High susceptibility was observed with S. agalactiae; this also has been noted before (7). The other gram-positive pathogens showed markedly similar susceptibilities.

TABLE 1. Minimum inhibitory concentration[a] of nisin on mastitis-causing organisms

Strain	ug nisin per ml
Enterococcus faecalis subsp. liquefaciens 27959	250
Staphylococcus aureus 29740	50
Staphylococcus epidermidis 14990	100
Streptococcus agalactiae 27956	10
Streptococcus equinus 27960	50
Streptococcus dysgalactiae 27957	100
Streptococcus uberis 27958	100
Escherichia coli V517	none observed

[a]Minimum nisin concentration showing a 13.5 mm zone of inhibition to organism after 24 h growth at 37°C on BHI containing 0.5% yeast extract.

Milk assays. Results for the milk assays (Table 2) indicated that all gram-positive species tested were strongly inhibited at 50 ug of nisin per ml. This concentration was lower than some of the MICs determined on the disc assay. The difference between the milk and disc assay data may be a result of the homogeneous distribution of nisin in liquid. Diffusion of nisin from the discs into the agar during the disc assay would diminish the actual concentration near the disc, thus resulting in higher MIC.

Table 2 demonstrates that nisin retained activity in milk, although Staph. aureus did show a small increase in cell numbers at 24 h. These cells may represent resistant

TABLE 2. Antimicrobial effect of 50 ug nisin per ml in milk on mastitis-causing organisms

Strain	nisin conc.	CFU/ml at t= 0 h	6 h	24 h
Staphylococcus aureus 29740	0	4.4×10^6	1.8×10^8	6.6×10^8
	50	4.9×10^6	0[a]	EAPC[b] 30
Staphylococcus epidermidis 14990	0	1.2×10^6	1.2×10^8	2.8×10^8
	50	1.4×10^6	EAPC[b] 30	EAPC[b] 2.5
Streptococcus agalactiae 27956	0	2.5×10^5	3.1×10^8	7.2×10^7
	50	6.5×10^5	0[a]	0[a]
Streptococcus dysgalactiae 27957	0	2.3×10^6	2.9×10^8	4.5×10^8
	50	2.7×10^6	0[a]	0[a]
Escherichia coli V517	0	5.2×10^6	4.7×10^8	5.8×10^8
	50	7.5×10^6	3.5×10^8	5.8×10^8

[a]None detected.
[b]Estimated aerobic plate count.

mutants or be indicative of slightly decreased nisin activity in milk (11,15).

In conclusion, mastitis is a significant dairy problem that continues to draw the attention of researchers seeking to improve current modes of control. Although disc assays may not reflect actual MIC values, results indicate that nisin is effective for inhibition of gram-positive, mastitis-causing bacteria in vitro. In vivo studies need to be performed to determine whether nisin would be a useful therapeutic agent for treatment of mastitis caused by gram-positive organisms. Nisin would not be useful for treating mastitis involving gram-negative pathogens.

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CHAPTER VI

CONCLUSION

The major focus of this work has been the development of nisin-producing dairy starter cultures. Through this focus we sought to support and expand an important application of biotechnology in the dairy foods industry: the employment of lactic bacteriocins as natural food preservatives. Bacteriocins are found among both gram-positive and gram-negative species and in general, these molecules exert a bactericidal effect only toward closely related species of bacteria. Bacteriocins produced by some gram-positive bacteria, including those employed in dairy fermentations, sometimes exhibit much broader spectra of antagonism. These molecules may act not only against related species but also against unrelated pathogenic or spoilage bacteria and even fungi. Production of bacteriocins has been demonstrated in every genera of lactic acid bacteria (3,8,12). Because of their proteinaceous nature, bacteriocins are degraded by stomach enzymes if they are consumed. The unique physical and inhibitory properties of the latter compounds has generated considerable interest toward their application as food preservatives. Although nisin is by far the most successful example of a bacteriocin with applications for food preservation, it is not unique to this application.

Microgard™ (Wesman Foods Inc., Beaverton, OR, USA) is the trade name given to a commercial preparation of a bacteriocin which has found successful application as a preservative for cottage cheese in the United States (4). Produced by a strain of Propionibacterium freudenreichii subsp. shermanii, the active compound in Microgard™ inhibits gram-negative bacteria and fungi but not gram-positive organisms (1). Lactic bacteriocins with relatively narrow spectra of activity may also prove useful for food preservation. A number of these compounds have been identified within members of the genus Lactobacillus (12) which contains species important to both food fermentation and spoilage (11). Bacteriocins inhibitory only to the lactobacilli may be quite useful in high acid products where spoilage by these microorganisms is predominant.

Future applications for these inhibitors will likely include an expansion of the number of bacteriocins available as commercial preparations for utilization in processed foods. In addition, genetics studies should eventually permit the construction of starter culture systems which synthesize one or more bacteriocins for enhanced preservation of fermented foods. Although the purification of some bacteriocins in a form which retains activity may pose an occasional obstacle to the development of new commercial preparations, construction and regulatory

approval of microorganisms for the latter application will prove to be the greater challenge. The discovery and development of gene transfer systems during the past 10 years presents investigators with the technology required to meet these challenges and expand the applications for "built in" food preservation mechanisms.

The prospects for construction of bacteriocin-producing starter cultures appears optimistic; in addition to genes involved in nisin production, genes which encode other lactic bacteriocins have been located upon plasmid or chromosomal DNA (12) and a few have subsequently been cloned and sequenced (9,10,16,17). Cocconcelli et al. (2) recently reported heterologous expression of the Pediococcus pentosaceous bacteriocin Pediocin A in an electro-transformed strain of Lactobacillus reuteri. In addition, several bacteriocin genes have been located upon conjugative plasmids or transposons (5,7,12), which may facilitate their distribution to other organisms.

As outlined in Chapter I, conjugally improved strains may also encounter fewer regulatory obstacles than strains which contain recombinant DNA molecules. This is important because, at present, one of the primary obstacles to the utilization of genetically improved starter cultures is the regulatory uncertainty which surrounds their application in food. At present, the FDA position toward food grade bacteria which have been transformed with intact plasmid

DNA isolated from other food grade bacteria is unclear. Conjugation has already been utilized to genetically improve strains for the dairy industry. Although the dairy industry must pursue FDA endorsement of transformation as a technique for strain improvement, the "case by case" approach of FDA (6) will probably delay widespread acceptance and application of this technology within the industry. For this reason conjugation will likely continue to be an important technique to genetically improve dairy starter cultures.

As demonstrated in Chapter III, conjugation facilitated the genetic construction of nisin-producing variants from fast acid-producing strains of L. lactis subsp. cremoris, the organism most commonly used to manufacture Cheddar-type cheeses. Characterization of the nisin-producing L. lactis subsp. cremoris transconjugants indicated that these strains retained the recipient traits which were important to milk fermentation. Those results suggested that conjugation could be used to construct nisin-producing strains of L. lactis subsp. cremoris for application in mesophilic starter systems with nisin-producing strains of L. lactis subsp. lactis. These starter systems should inhibit undesirable gram-positive bacteria yet retain the capability, which is associated with L. lactis subsp. cremoris starters, to produce quality cheese.

Further potential for conjugal construction of nisin-producing starters was demonstrated in Chapter IV by the intergeneric transfer of nisin genes from Lactococcus lactis subsp. lactis to Streptococcus salivarius subsp. thermophilus recipients. Although these transconjugants did not produce nisin, nisin immune strains could also function as part of a starter blends which included nisin producers. S. salivarius subsp. thermophilus are sometimes used with lactococci in Cheddar cheese as a safety measure to ensure successful acid production in the event of a lactococcal phage attack (J. Kondo, Marschall Products, pers. comm.). Consequently, nisin-immune S. salivarius subsp. thermophilus could be utilized with the nisin-producing blends of L. lactis subsp. cremoris just described, to simultaneously guard against phage attack and obtain the benefits of nisin production. Although intergeneric exchange of nisin genes was demonstrated, further studies will be necessary to achieve this result with all genera of lactic acid bacteria. It would be desirable, for example, to develop nisin-producing or nisin-immune cultures of Lactobacillus helveticus or lactobacillus delbruekii subsp. bulgaricus. These organisms are commonly paired with strains of S. salivarius subsp. thermophilus in the manufacture of Swiss and Italian cheeses or yogurt, so nisin-producing or -immune lactobacilli would facilitate the inclusion of nisin in

these products.

As discussed in Chapter III, commercial utilization of the transconjugants constructed in this study would first require that they be cured of the antibiotic resistance plasmid that was utilized for conjugal selection. Once accomplished, it may be prudent for the culture supplier to notify FDA of their intent to distribute the cultures and perhaps even provide the agency with data which demonstrated that the cultures were free of deleterious antibiotic resistance genes. Alternatively, it may be possible to utilize the NSD nisin donor strains constructed in Chapter IV to develop nisin-producing L. lactis subsp. cremoris strains using the strategy of Sanders et. al. (13). This approach would employ nisin resistance and the ability to ferment lactose (which NSD strains lack) to identify transconjugants. Transconjugants obtained in this manner would not require any regulatory approval.

The results presented in this work indicated that conjugation may be useful for the construction, from cultures which produce high quality dairy products, of nisin-producing starters with the capability to inhibit spoilage or pathogenic microorganisms associated with dairy products. Widespread and specific application of nisin and other natural food preservatives may be envisioned as more bacteriocins and the genes which control their synthesis are identified, isolated and characterized.

One potential barrier to this and other applications for biotechnology in the dairy culture industry involves consumer misperception. With respect to this particular application, perpetuation of terminology which describes nisin and other broad-spectrum bacteriocins as antibiotics may lead to unfounded concerns among consumers about the presence of these proteins in food. Antibiotics in food are a legitimate concern thus care must be taken to ensure consumers do not confuse harmless proteinaceous bacteriocins with dangerous drugs.

Finally, the broad spectrum of inhibition nisin exhibits toward gram-positive organisms indicated that nisin may have medicinal applications where gram-positive bacteria present a problem. Results presented in Chapter V suggested that one potential application may be the control or prevention of bovine mastitis. This possibility was recently supported by the introduction of a nisin-based commercial teat dip formulation for the prevention of bovine mastitis (14,15).

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APPENDICES

APPENDIX A

DATA NOT INCLUDED IN TEXT

FIG. 1. Examples of plasmid analysis data for Nip$^-$Suc$^-$ recipients and their Nip$^+$Suc$^+$ transconjugants. Lanes 1-6 of part A contain plasmid DNA isolated from Nip$^+$Suc$^+$ transconjugants of recipient strain L. lactis subsp. lactis KG491, which is shown in lane 7. The donor strain for this mating was L. lactis subsp. lactis 11454, shown in lane 8. 11454 also served as the donor for the plasmid-free recipient L. lactis subsp. lactis LM2306, shown in lane 1 of part B, and for the recipients included in part C. Lanes 2-7 of part B contain Nip$^+$Suc$^+$ transconjugants of LM2306, and lane 8 contains plasmid size standards isolated from E. coli V517. Part C contains plasmid DNA isolated from two L. lactis subsp. lactis Nip$^-$Suc$^-$ recipients; JB0213 in lane 1, and JBR13 in lane 7. Examples of the plasmid profile observed for Nip$^+$Suc$^+$ transconjugants of JB0213 are shown in lanes 2 and 3 while lanes 4-6 reveal the plasmid profile noted among transconjugants of JBR13. Lanes 3 and 4 of part D contain plasmid DNA isolated from Suc$^+$Nip$^-$ transconjugants of L. lactis subsp. lactis recipient JK2301β (lane 5), which contains pAMβ1, and L. lactis subsp. lactis Suc$^+$ donor DL16 (lane 2). Lane 1 of part D contains V517 plasmid size standards.

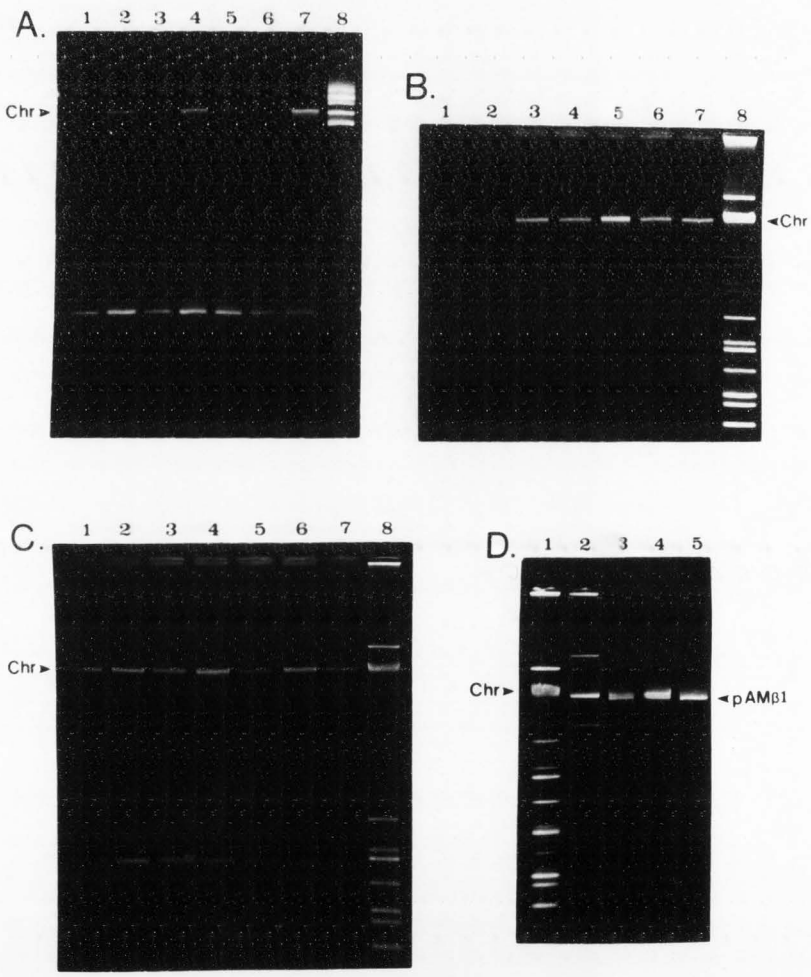
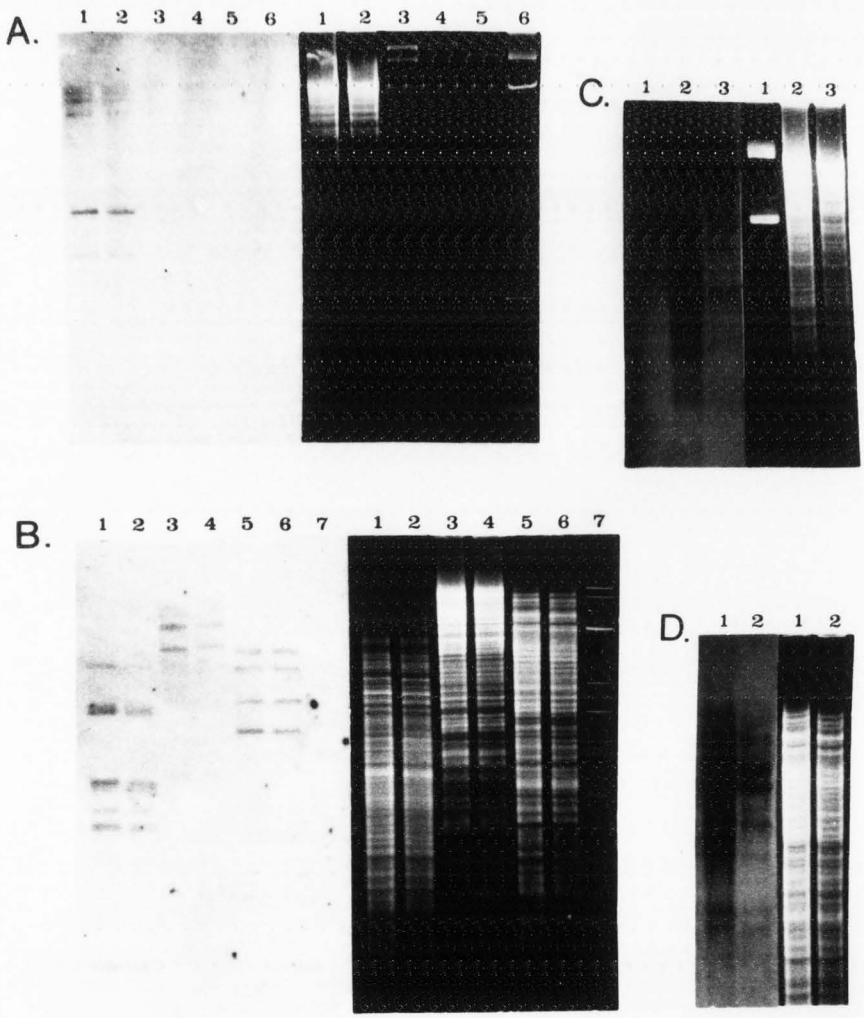

A.
1 2 3 4 5 6 7 8
Chr ►
B.
1 2 3 4 5 6 7 8
◄Chr
C.
1 2 3 4 5 6 7 8
Chr ►
D.
1 2 3 4 5
Chr ►
◄pAMβ1

FIG. 2. Hybridization of Nip^+Suc^+ and Nip^-Suc^- lactococcal DNA to *Streptococcus mutans* sucrose gene probes. The left half of each figure shows the autoradiogram obtained after high-stringency hybridization to DNA samples taken from the agarose gels on the right half of each figure. Autoradiograms in parts A and B were obtained after 72-h exposures while those in C and D were obtained after 48-h. Parts A and B show the results of lactococcal DNAs probed with pMH613. Lanes 1 and 2 of part A contain *Kpn*1 restricted genomic DNA isolated from the plasmid-free Nip^-Suc^- recipient strain, LM2306, and a Nip^+Suc^+ transconjugant of that strain, NS5406. Lanes 3-5 contain plasmid DNA isolated from Nip^+Suc^+ parental strains DL16, 7962, and 11454 and lane 6 contains *E. coli* V517 plasmid size standards. Lanes 1 and 2 of part B hold *Hin*dIII restricted genomic DNA isolated from LM2306 and NS5406, lanes 3 and 4 are LM2306 and NS5406 cut with *Cla*I, and lanes 5 and 6 contain *Eco*RI digests of LM2306 and NS5406 DNA. Lane 7 holds *Bam*HI/*Eco*RI digested Adenovirus 2 DNA fragment size standards. Parts C and D show results of probing genomic DNAs with a 1.9 kb *Pvu*II fragment of pMH613 that contained most of the *S. mutans scrA* gene. Lane 1 in part C contains $CsCl_2$-purified pMH613, and lanes 2 and 3 are *Kpn*I digests of LM2306 and NS5406 genomic DNA. Lanes 1 and 2 of part D contain *Eco*RI digests of LM2306 and NS5406 genomic DNA.


A.
1 2 3 4 5 6 1 2 3 4 5 6
B.
1 2 3 4 5 6 7 1 2 3 4 5 6 7
C.
1 2 3 1 2 3
D.
1 2 1 2

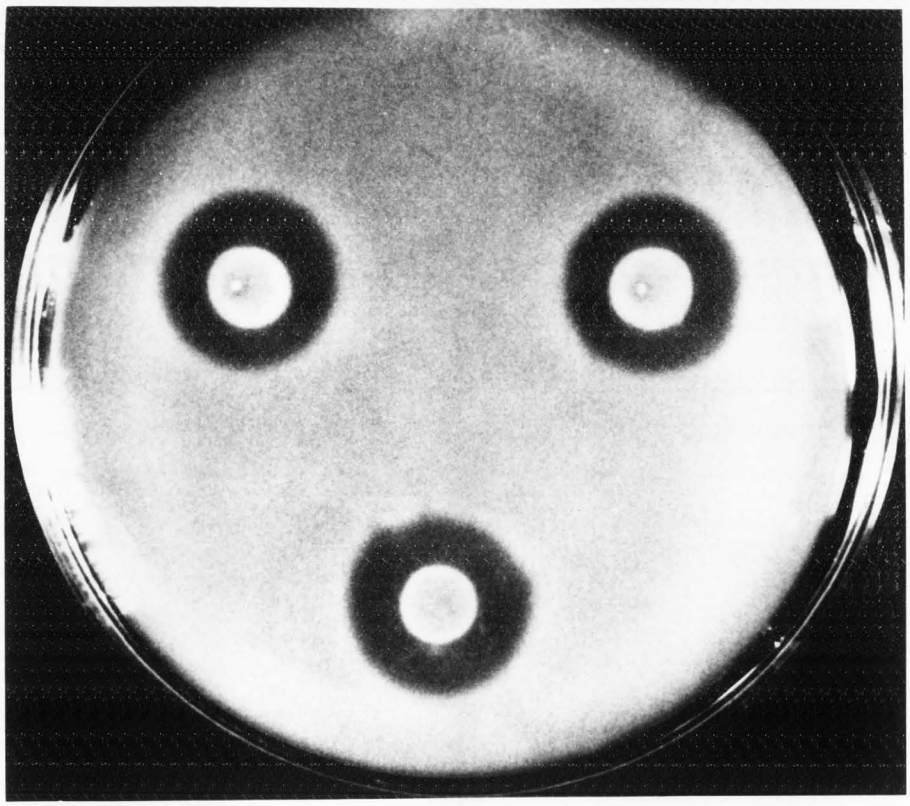


FIG. 3. Example of an agarose overlay assay for nisin production among Suc$^+$ transconjugants of L. lactis subsp. lactis LM2306. Three erythromycin-resistant, Suc$^+$ transconjugants were assayed upon each agar plate. As shown in the figure, nisin production results in a zone of indicator strain (LM2306) inhibition around the Nip$^+$ colony.

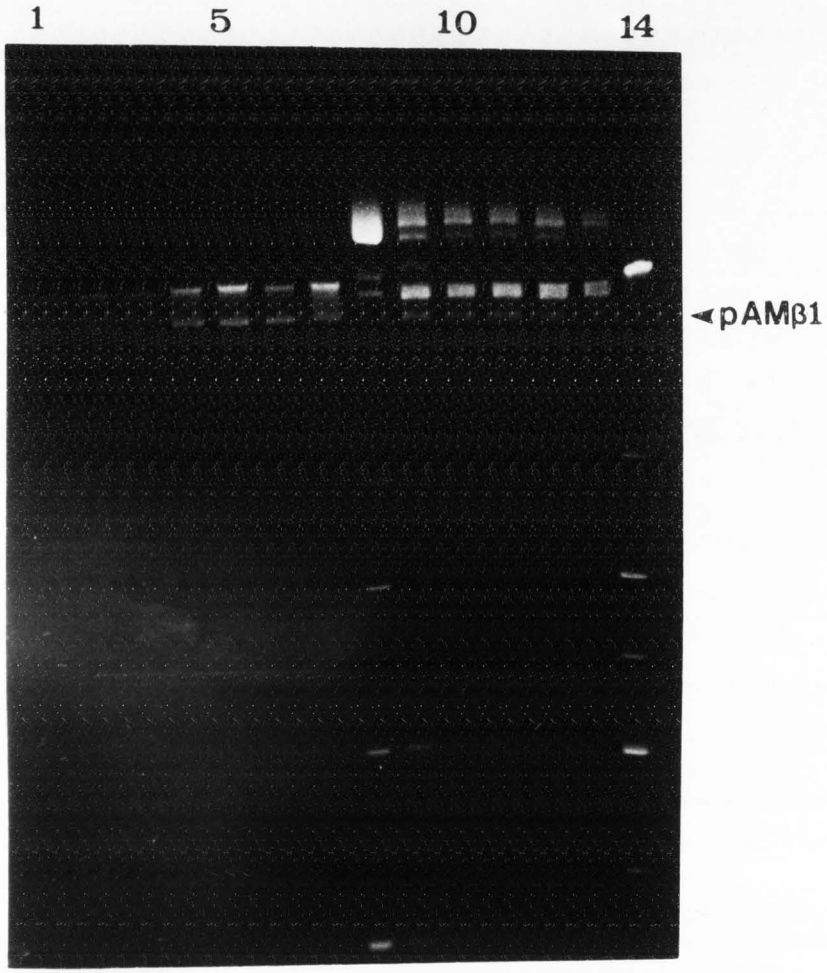


FIG. 4. Plasmid profiles of Suc^+, erythromycin-resistant transconjugants isolated from DPC and milk agar matings between L. lactis subsp. lactis recipient JK2301β and Nip^+Suc^+ donor 11454. Lanes 1-3 contain plasmid DNA isolated from Suc^+Em^r transconjugants obtained from DPC matings. All transconjugants from DPC matings were Lac^-. Lanes 4-6 contain plasmid DNA isolated from $Lac^-Suc^+Em^r$ transconjugants obtained from milk agar conjugations. Recipient strain JK2301β is shown in lane 7, and donor strain 11454 is present in lane 8. Lanes 9-13 contain plasmid DNA isolated from $Lac^+Suc^+Em^r$ transconjugants collected from milk agar conjugations, and lane 14 contains plasmid size standards isolated from E. coli V517.

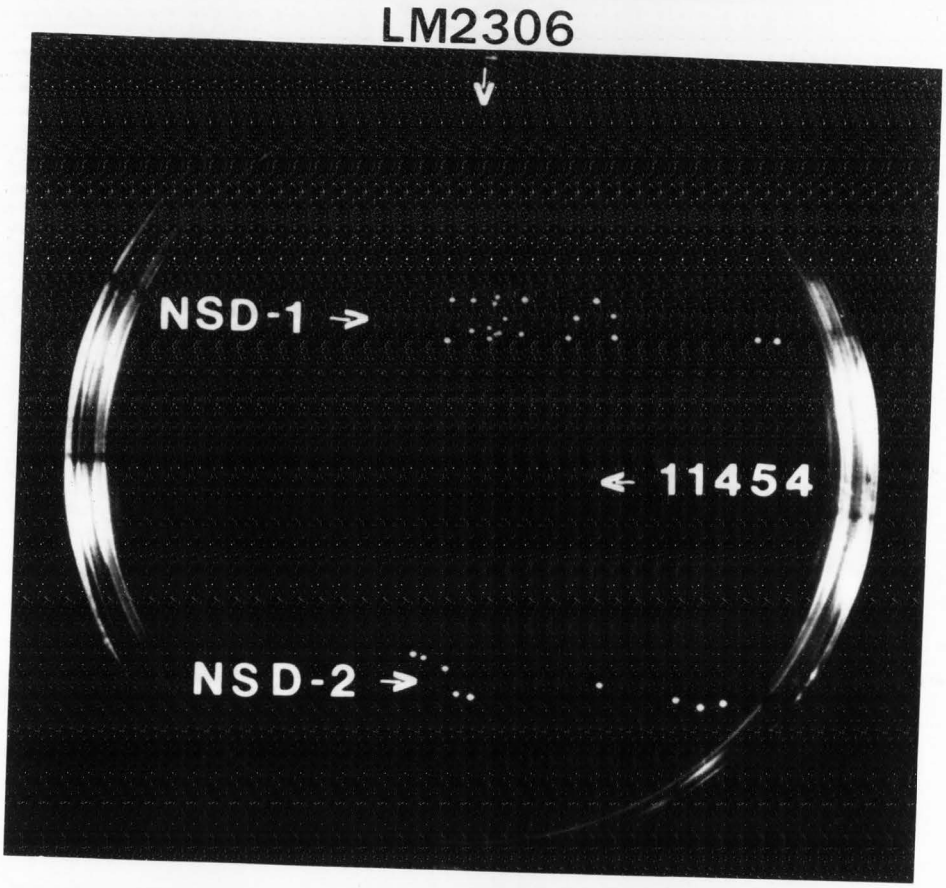
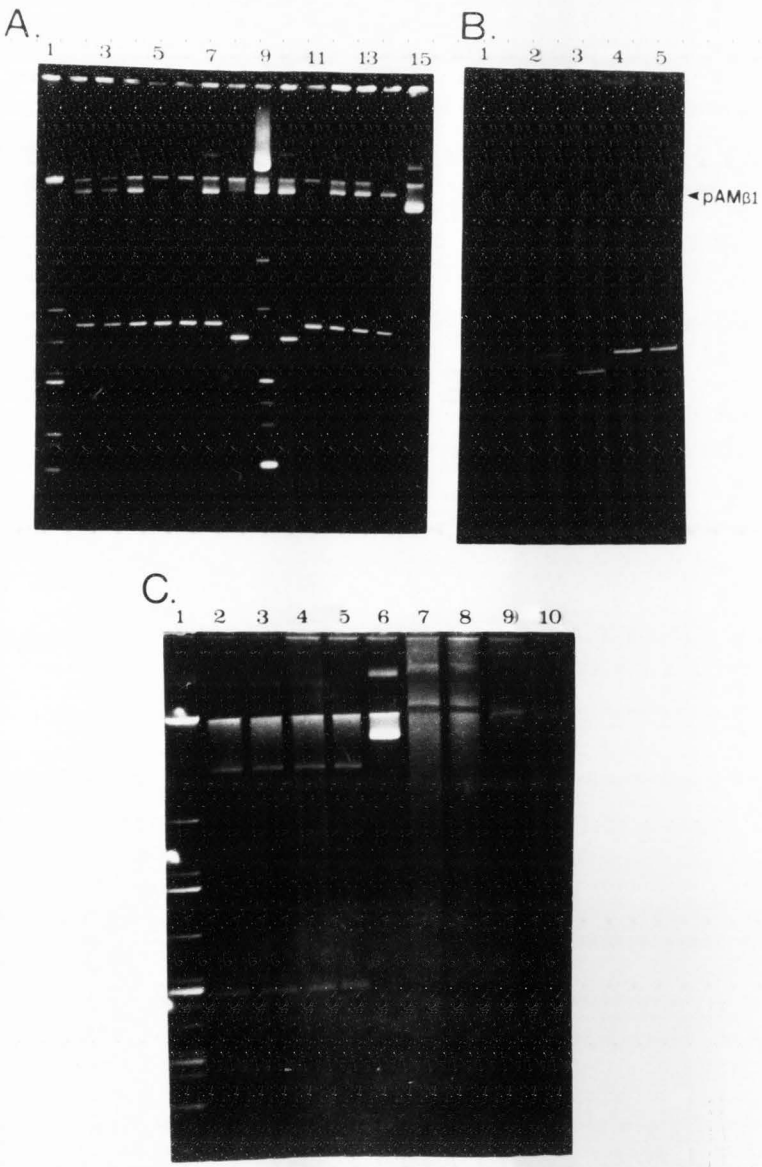


FIG. 5. Cross streak assay for high-frequency Nip^+Suc^+ transfer between L. lactis subsp. lactis donors NSD-1, NSD-2, and 11454, to recipient LM2306. Arrows next to strain designations indicate the direction that cells were streaked with sterile cotton applicators. LM2306 was streaked first and the prospective Nip^+ donors were streaked perpendicular to the recipient. The plate was BCP-sucrose agar which contained 7 ug per ml of erythromycin. The white dots are Suc^+, erythromycin-resistant transconjugant colonies which appeared after 48 h of incubation at 30°C.

FIG. 6. Agarose gel electrophoresis of plasmid DNA isolated from Leuconostoc spp. and Streptococcus salivarius subsp. thermophilus parental strains and transconjugants which acquired pAMβ1. Part A contains plasmid DNA isolated from Streptococcus salivarius subsp. thermophilus parental strain S3 in lane 1. Shown in lanes 2-5 are four erythromycin-resistant transconjugants of S3, and lane 6 contains $CsCl_2$-purified pAMβ1. Lane 1 of Part B contains E. coli V517 plasmid size standards. Lanes 2-7 of part B contain erythromycin-resistant transconjugants of Streptococcus salivarius subsp. thermophilus parental strain S12, which is shown in lane 8. Part C contains plasmid DNA isolated from Leuconostoc spp. Lane 1 contains V517 plasmid size standards, lanes 2 and 3 each contain plasmid DNA isolated from Leuconostoc mesenteroides subsp. cremoris parental strain 44-4, and lanes 4 and 5 contain erythromycin-resistant transconjugants of 44-4. Lane 6 contains $CsCl_2$-purified pAMβ1, lanes 7 and 8 contain plasmid DNA isolated from erythromycin-resistant transconjugants of Leuconostoc mesenteroides subsp. dextranicum parental strain 181, which is shown in lanes 9 and 10.


A.
1 3 5 7 9 11 13 15
B.
1 2 3 4 5
◄pAMβ1
C.
1 2 3 4 5 6 7 8 9 10

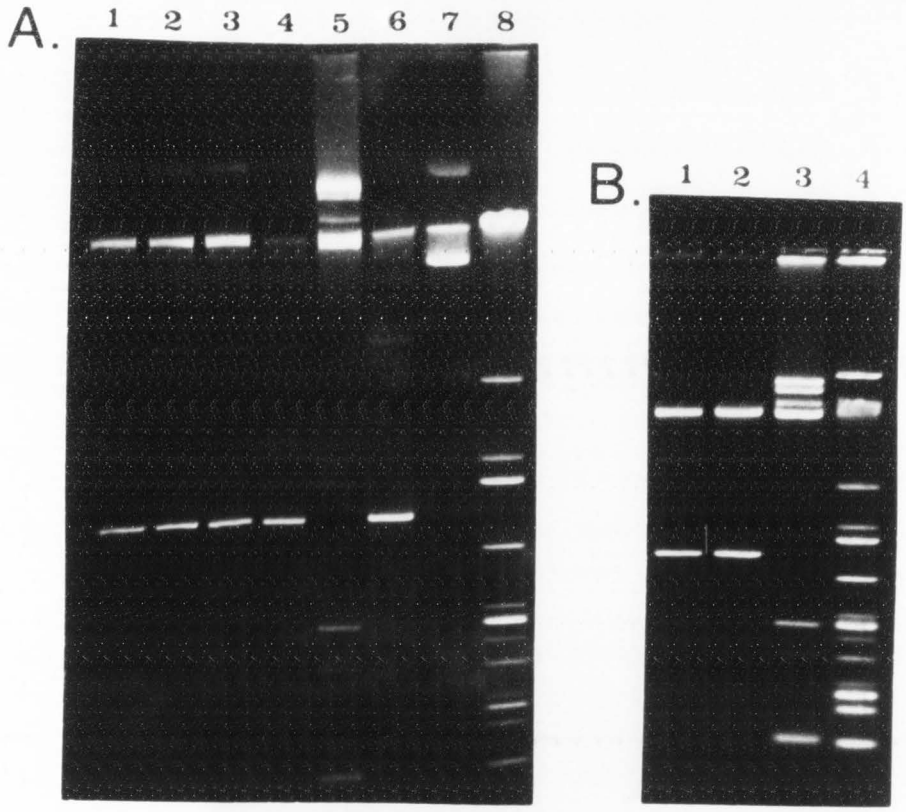


FIG. 7. Agarose gel electrophoresis of plasmid DNA isolated from Streptococcus salivarius subsp. thermophilus recipients which contained pAMβ1 and their Suc^+ transconjugants. Lane 1 of part A contains plasmid DNA isolated from recipient S12β, lanes 2 and 3 are Suc^+ transconjugants of S12β (NIS12β) and lanes 4-6 contain plasmid DNA isolated from recipient S3β, the L. lactis subsp. lactis Suc^+ donor 11454, and a Suc^+ transconjugant of S3β (NIS3β). Shown in lanes 7 and 8 are $CsCl_2$-purified pAMβ1 and E. coli V517 plasmid size standards. Lanes 1 and 2 of part B show the plasmid content of recipient S4β and a Suc^+ transconjugant of S4β (NIS4β). Plasmid DNA isolated from donor strain 11454 is in lane 3 and lane 4 contains V517 plasmid size standards.

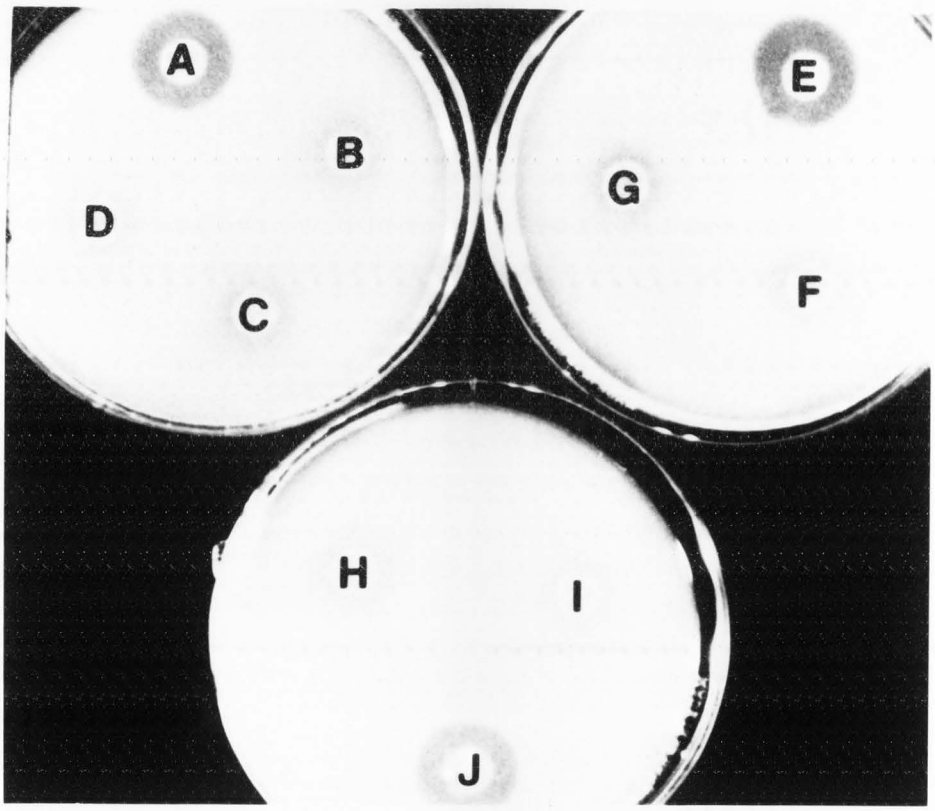


FIG. 8. Assay for nisin production in $Suc^{+}Nis^{r}$ transconjugants of S. salivarius subsp. thermophilus. Spots A, E, and J are colonies of the L. lactis subsp. lactis $Nip^{+}Suc^{+}$ donor strain 11454, spots B, F, and H are the Suc^{-} recipients S4β, S3β, and S12β, D and C are both $Suc^{+}Nis^{r}$ transconjugants of S4β (NIS4β), colony G is a $Suc^{+}Nis^{r}$ transconjugant of S3β, NIS3β, and I is NIS12β, a $Suc^{+}Nis^{r}$ transconjugant of S12β.

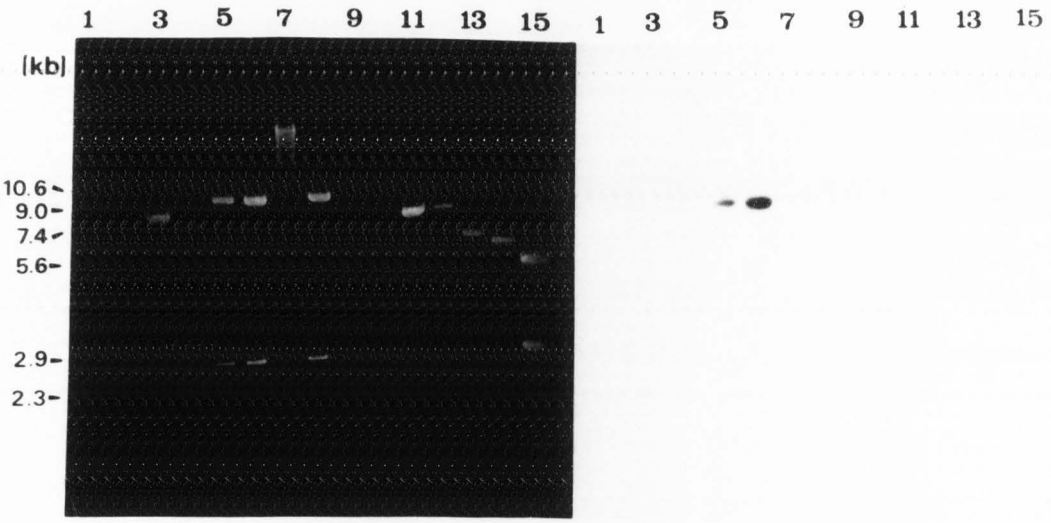


FIG. 9. Identification of E. coli JM109 clones which contained the nisin structural gene, nisA. Plasmid DNA was isolated from clones which contained putative recombinant molecules and restricted with KpnI. The samples were separated in the agarose gel shown in the left half of the figure, transferred to nitrocellulose, and probed with the nisA-specific oligonucleotide. DNA samples included in the gel are; Raoul™ I fragment size standards in lane 1, lanes 2-6 contain clones A-E, lane 7 contains KpnI restricted genomic DNA isolated from the $Nip^{+}Suc^{+}$ transconjugant NS5406 (positive control), and lanes 8-15 contain plasmid DNA isolated from clones F-M. The autoradiogram shown in the right half of the figure was obtained after a 24 h exposure, and demonstrated that clones D and E contained the nisA gene (72 h exposures were required to detect hybridization by the positive control; e.g., Chapter II, Fig. 3.).

TABLE 1. Effects of RNase A, nuclease S1, and restriction endonuclease MspI on Nip^+Suc^+ transfer frequency[a]

Treatment	Transfer Frequency
RNase A:	
1.0×10^3 U RNase A per ml[b]	2.4×10^{-6}
Control	2.4×10^{-6}
Nuclease S1:	
8.3×10^2 U nuclease S1 per ml[c]	1.4×10^{-5}
Control	1.7×10^{-5}
Restriction endonuclease MspI:	
60 U MspI per ml[d]	3.6×10^{-6}
Control	3.9×10^{-6}

[a]L. lactis subsp. lactis 11454 x L. lactis subsp. lactis LM2306. Enzymes were included in mating mixture at concentrations indicated above. Control matings were performed with cells suspended in the respective buffers without added enzyme.
[b]In 0.85% saline.
[c]In supplier (United States Biochemical, Cleveland, Ohio) recommended buffer (50 mM sodium acetate [pH 4.6], 1 mM $ZnCl_2$, 250 mM NaCl, 50 ug per ml bovine serum albumin).
[d]In reaction buffer (25 mM Tris-HCl [pH 7.8], 50 mM NaCl, 10 mM $MgCl_2$, 100 ug per ml of bovine serum albumin, 2 mM β-mercaptoethanol) supplied by International Biotechnologies Inc., New Haven, Conn.

APPENDIX B

COPYRIGHT PERMISSION FORMS

UTAH STATE UNIVERSITY • LOGAN, UTAH 84322-8700

Department of Nutrition and Food Sciences
College of Agriculture
College of Family Life
Telephone (801) 750-2126
FAX (801) 750-2379

Linda Illig
American Society for Microbiology
1325 Massachusetts Ave., N.W.
Washington, D.C. 20005-4171

April 17, 1992

Dear Ms. Illig,

I am in the process of preparing my dissertation in the Department of Nutrition and Food Science at Utah State University. I will complete in June of this year.

I am requesting permission from ASM to include a copy of my manuscript which was published in the February 1991 issue of Applied and Environmental Microbiology. The paper is entitled "Genetic construction of nisin-producing Lactococcus lactis subsp. cremoris and analysis of a rapid method for conjugation". Please send me a letter to indicate ASM approval of this request and to indicate whether any reprint fees are involved. My address is:

Jeffery R. Broadbent
Dept. NFS
Utah State University
Logan, UT 84322-8700

If you have any questions please call me at (801) 750-2113. Thank you for your assistance and cooperation.

Sincerely,

Jeffery R. Broadbent

PERMISSION GRANTED CONTINGENT ON AUTHOR PERMISSION
AND APPROPRIATE CREDIT
American Society for Microbiology
Journals Division

Date 4-20-92

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To Jeff Broadbent	From C. Nimz
Co.	Co. ADSA
Dept.	Phone #
Fax # 801-750 2319	Fax #

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CURRICULUM VITAE

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Ph.D.

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Utah State University
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PERSONAL:

Married, interests include falconry and snow skiing.
Birthdate: April 19, 1961
Birthplace: Payson, Utah

EDUCATION:

Degree	Year	Institution
B.S., Cum Laude	1987	Utah State University Logan, UT 84322

Major: Biology with biochemistry emphasis.

Minor: Chemistry.

3.51 GPA

Ph.D.	1992	Utah State University Logan, UT 84322

Major: Nutrition and Food Sciences with focus upon genetics and microbiology of lactic acid bacteria.

Dissertation title: Genetics and applications of nisin production from Lactococcus lactis subsp. lactis.

3.96 GPA

HONORS:

1990 First-place winner of American Dairy Science Association Dairy Foods Division graduate student scientific paper presentation competition.

Recipient of a Provost summer fellowship from U.S.U.

1989 Recipient of the Clara L. Budge Scholarship from U.S.U. College of Family Life.

1988 Recipient of a three-year, Food and Agricultural Sciences National Needs Graduate Fellowship from the United States Department of Agriculture-Agricultural Research Services.

1987 Graduated Cum Laude with **B.S.** degree.

1986 Recipient of the Christensen Memorial Scholarship from U.S.U. Department of Biology.

RESEARCH EXPERIENCE:

Position: Senior laboratory technician, Aug. 1987-June 1988.
Ph.D. candidate, June 1988-present.

Employer: Dr. Jeffery K. Kondo, Associate professor
Department of Nutrition and Food Sciences,
Utah State University, Logan, UT 84322-8700.

Duties and Responsibilities:

Designed and performed experiments related to Western Dairy Foods Research Center Grant which required genetic manipulation of Leuconostoc spp.

Utilized and developed modern genetics techniques for lactic acid bacteria.

Wrote, submitted, and presented scientific papers, and progress reports which described original research.

Wrote a Western Dairy Foods Research Center grant proposal.

Reviewed research manuscripts written by investigators within and outside of the Nutrition and Food Sciences Department at Utah State University.

Assumed responsibility for all aspects of laboratory operations after the departure of Dr. Jeffery Kondo on August 25, 1990.

TEACHING EXPERIENCE:

Co-instructor, Nutrition and Food Sciences 614[a]: Biotechnology of Lactic Starter Cultures, Spring 1991. Dept. of Nutrition and Food Sciences, Utah State University, Logan, UT 84322-8700.

Instructor, Microbiology 420[b]: Recombinant DNA Techniques, Spring 1991. Dept. of Microbiology, 2506 Weber State University, Ogden, UT 84408-2506.

Co-instructor, Microbiology 512[a]: Food Fermentations, Winter 1991. Dept. of Biology, Utah State University, Logan, UT 84322-5305.

Co-instructor, Workshop on Biotechnology, May 11, 1990. Weber State University, Ogden, UT 84408.

Teaching assistant, Microbiology 513: Food Fermentations laboratory, Winter 1988. Dept. of Biology, Utah State University, Logan, UT 84322-5305.

Laboratory instructor, Biology 127: Animal Biology, Spring 1987. Dept. of Biology, Utah State University, Logan UT 84322-5305.

---

[a]Results from student evaluations of teaching effectiveness at Utah State University:

1 to 4 rating for overall course relevance (1 = disagree strongly; 4 = agree strongly).

NFS 614:	3.13/4.00
Micro 512:	3.39/4.00

1 to 10 rating for overall teaching effectiveness (1 = worst; 10 = best).

NFS 614:	7.33/10.00
Micro 512:	7.13/10.00

[b]Results from student evaluations of teaching effectiveness at Weber State University:

1 to 7 rating (1 = worst; 7 = best) of Micro 420.

Overall course appraisal:	6.3/7.0
Overall rating of instructor:	6.5/7.0

REFEREED PUBLICATIONS:

Broadbent, J.R., and J.K. Kondo. 1991. Genetic construction of nisin-producing Lactococcus lactis subsp. cremoris and analysis of a rapid method for conjugation. Appl. Environ. Microbiol. 57:517-524. (Dissertation results).

Broadbent, J.R., Y.C. Chou, K. Gillies, and J.K. Kondo. 1989. Nisin inhibits several gram-positive mastitis-causing pathogens. J. Dairy Sci. 72:3342-3345. (Dissertation results).

Broadbent, J.R., J.K. Kondo, and W.E. Sandine. 1992a. Physiological characteristics of nisin-sucrose conjugal transfer and intergeneric exchange of genes associated with nisin production. Appl. Environ. Microbiol. (Submitted dissertation results).

Broadbent, J.R., J.K. Kondo, and W.E. Sandine. 1992b. Location of the nisin genes in the producer organism. J. Food Protect. (Submitted dissertation results).

Oberg, C.J., and J.R. Broadbent. 1992. Thermophilic starter cultures: another set of problems. J. Dairy Sci. (Submitted review manuscript).

BOOK CHAPTER:

Broadbent, J.R., and J.K. Kondo. 1992. Biotechnology of dairy lactic acid bacteria. In Y.H. Hui (ed.), Dairy science and technology, vol. 3: applications science, technology and engineering. VCH Publishers Inc., New York, NY. (Invited review).

ABSTRACT:

Broadbent, J.R., and J.K. Kondo. 1990. Genetic construction of nisin-producing strains of Lactococcus lactis ssp. cremoris. J. Dairy Sci. 73(Suppl. 1):72.

NON-REFEREED PUBLICATIONS:

Kondo, J.K., W.E. Sandine, J.R. Broadbent, and H. Wycoff. 1988-1991. Cloning the nisin and other genes of lactic streptococci into Leuconostoc species and amplification of nisin production. Proc. Ann. Mtg. Western Dairy Foods Research Center.

GRANT PROPOSAL:

Kondo, J.K. 1990. Use of genetically engineered nisin-producing Lactococcus lactis subsp. cremoris to inhibit spoilage and pathogenic bacteria in mixed and multiple starter culture milk fermentations. Submitted to Western Dairy Foods Research Center (students are not permitted to submit grants).

PROFESSIONAL PRESENTATIONS:

Broadbent, J.R. 1992. Biotechnology in dairy microbiology and recombinant DNA. Weber State University American Society for Microbiology lecture series.

Kondo, J.K., W.E. Sandine, J.R. Broadbent, and H. Wycoff. 1991. Cloning and intergeneric conjugal exchange of genes associated with nisin production. Ann. Mtg. Western Dairy Foods Research Center.

Broadbent, J.R., and J.K. Kondo. 1990. Genetic construction of nisin-producing strains of Lactococcus lactis subsp. cremoris. Ann. Mtg. American Dairy Science Association. Placed first in Dairy Foods Division graduate student paper competition.

Kondo, J.K., W.E. Sandine, J.R. Broadbent, and H. Wycoff. 1990. Use of direct-plate conjugation to construct nisin-producing strains of Lactococcus lactis subsp. cremoris. Ann. Mtg. Western Dairy Foods Research Center.

Kondo, J.K., W.E. Sandine, J.R. Broadbent, and H. Wycoff. 1989. Development of the direct-plate conjugation technique for nisin transfer. Ann. Mtg. Western Dairy Foods Research Center.

Kondo, J.K., W.E. Sandine, J.R. Broadbent, and H. Wycoff. 1988. Development of electro-transformation in Leuconostoc spp. and analysis of nisin production among plasmid cured derivatives of Lactococcus lactis subsp. lactis 7962. Ann. Mtg. Western Dairy Foods Research Center.

PROFESSIONAL ACTIVITIES:

1991 Chair, microbiology section, Western Dairy Foods Research Center annual meeting, Logan, UT.

1991 Reviewed a research manuscript for the editor of the Journal of Food Protection.

1986 Undergraduate representative to U.S.U. Department of Biology review of B.S. degree requirements.

MEMBERSHIP IN PROFESSIONAL ORGANIZATIONS:

American Dairy Science Association

American Society for Microbiology

Institute of Food Technologists

ATTENDANCE AT PROFESSIONAL MEETINGS:

1991 American Dairy Science Association Annual Meeting. Logan, UT, USA.

Western Dairy Foods Research Center Annual Meeting. Logan, UT, USA.

1990 American Society for Microbiology Third International Symposium on Streptococcal Genetics. Minneapolis, MN, USA.

American Dairy Science Association Annual Meeting. Raleigh, NC, USA.

Western Dairy Foods Research Center Annual Meeting. Corvallis, OR, USA.

1989 Western Dairy Foods Research Center Annual Meeting. Logan, UT, USA.

1988 American Dairy Science Association Annual Meeting. Edmonton, Alberta, Canada.

Western Dairy Foods Research Center Annual Meeting. Logan, UT, USA.

MEMBERSHIP IN HONOR SOCIETIES:

Phi Kappa Phi Honor Society